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FLUORINE AND DENTAL CARIES

STUDIES ON THE EFFECTS OF FLUORINE
COMPOUNDS ON THE ORAL TISSUES IN RELATION
TO THE REDUCTION OF DENTAL CARIES
BY FLUORIDES

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CONTENTS

PREFACE.....	5
1. INTRODUCTION.....	6
2. THE PROBLEM.....	13
Effect of fluoride on caries	16
Mechanism of fluoride action	19
Chemical properties of hard tissues	21
Mechanism of fluoride incorporation	25
Investigation of reaction	33
Measurement of fluoride	34
Plan of study	37
3. POLAROGRAPHIC STUDIES.....	39
Separation of fluoride	49
Polarography of arsonic acids	60
Discussion	63
4. ENZYME STUDIES.....	68
Substrate	72
Measurement of enzyme activity	76
Enzyme preparation	81
Purification of enzyme	84
Properties of enzyme extracts	91
Kinetics	93
Product inhibition	98
Short incubation experiments	101
Analytical possibilities	111
Discussion	116
5. ENAMEL STUDIES.....	125
Intact enamel	125
Powdered enamel	130
Preparation of powder	132
Separation of enamel powder	133
Examination of enamel powder	137
Removal of enamel powder from suspensions	140
Uptake of fluoride	141
Time-course of fluoride uptake	145
Theory of fluoride uptake	154
Discussion	158

6.	CONCLUSION.....	161
7.	SUMMARY.....	164
	Acknowledgments	166
	Abbreviations	167
8.	REFERENCES.....	168
	Reports	168
	Authors	168
9.	APPENDIXES.....	187
	List of publications	187
	Reprints	

	<u>Figures</u>	facing page
1.	Calibration curve for polarograph	48
2.	Time-course of enzyme reaction	93
3.	Enzyme activity at different substrate concentrations	103
4.	Uptake of fluoride by sequential sampling	146
5.	Fluoride uptake by steamed bone	156
6.	Fluoride uptake over longer period	157

	<u>Tables</u>	
I.	Reproducibility of polarograph measurements	47
II.	Extinctions at specified wavelengths	78
III.	Effect of acid on enzyme reaction	80
IV.	Measurement of organic content	84
V.	Time-course of reaction	93
VI.	Calculated values for K and V	94
VII.	Reaction sequences leading to negative constants	98
VIII.	Effect of pH on enzyme activity	104
IX.	Effect of fluoride concentration	106
X.	Kinetics of fluoride inhibition	108
XI.	Reproducibility of enzymic determination	114
XII.	Density distribution of powdered teeth	134
XIII.	Densities of dentine	136
XIV.	Effect of centrifuging enamel suspension	140
XV.	Effect of various substances on esterase activity	148
XVI.	Initial fluoride uptake	150
XVII.	Fluoride uptake over longer period	154

Berzelius mentions that fluoride of calcium has been found in the waters of Carlsbad..... Middleton has found it in the London pipe-water.....I was induced to search for it in the water used in certain of the breweries in Edinburgh, in consequence of learning that these rapidly corrode the thermometers employed to regulate the temperature of the boilers and vats.....I visited the brewery of Mr. Campbell, situated in the Cowgate, behind Minto House, and was shewn by his manager a thermometer which.....had been in use only a few weeks, but was nevertheless so dimmed, that it required to be dipped into water in order to confer upon it a temporary transparency.....it is impossible not to connect the fact that the thermometers are corroded, with the circumstance that the water which occasions this corrosion contains fluoride of calcium.....

.....Water, as my own experiments prove, may carry away fluoride of calcium from osseous remains, as well as transport it to them. We require to account for its detention in bones, as well as for its conveyance to them. From an experiment made in the laboratory, as well as from their association in nature, I am inclined to think that there is a double phosphate of lime and fluoride of calcium, much less soluble than the latter salt is; and that the production of this compound fixes the fluoride and prevents its abstraction by water.

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CHAPTER 1

INTRODUCTION

Following the confirmation of the presence of fluorine in bone¹⁴⁷ in the nineteenth century, further work on the physiology of fluorine was limited by the inaccuracy of the methods of chemical analysis. By modern standards, the results quoted were very high, probably due to phosphate contamination. However, it appears that around 1860 a paper was published comparing the fluorine content of normal and rachitic bone, the latter being reported lower. The original article has never been located and is only known by implication⁵⁸. It appears that, since caries was considered to be a form of rickets at that time, this was the basis of the recommendation⁵² to give fluoride in the form of Hunter's pastilles.

By the turn of the century Gabriel, in a classical paper⁶⁵, which also introduced the procedure of ashing with alkaline glycerol, was able to assert that teeth normally contain less than 0.05% fluorine in the ash. It was not

until the use of steam distillation was introduced¹⁸⁶~~184~~, however, that it was possible to obtain reliable fluorine analyses.

It had been recognised early that fossils contained a relatively large amount of fluorine. In fact the first report¹³¹~~129~~ of fluorine in material of biological origin was concerned with enamel from a fossil elephant tooth. The source of the fluorine was the subject of some controversy until the investigations of Middleton¹²⁸~~126~~ who concluded that the fluorine was progressively taken up from the water surrounding the fossil, and that the amount of fluorine could be used as a measure of the age of the fossil. Thereafter, little interest was taken in the question for almost a century.

In fact it was a completely different approach which led to the revival of interest in the biochemistry of fluorine: a brown discoloration of the teeth which was found to occur extensively in certain parts of the Rocky Mountains was the subject of an extensive epidemiological investigation¹¹⁸~~116~~. It was shown to be sharply confined to certain regions and to depend on the type of water used, but

analyses of the water failed to show any differences.

The cause remained unknown until 1931 when analyses of water from these areas showed the presence of fluoride³⁷. At the same time it was found¹⁶⁶ that when the water was boiled down and fed to rats, mottling of the incisors could be produced, similar in appearance to the effects produced by administering fluoride¹¹⁶.

This led to work which initially was related to removal of the excess fluoride¹⁶⁵, but at the same time there was a growing realisation that the incidence of caries was low in areas with enamel fluorosis^{8,119}. The relationship could not be regarded as proved (more through reluctance to accept that teeth which appeared badly formed could be regarded as more resistant to caries than through lack of evidence) until the series of investigations by Dean on the relation of the fluoride concentration of the water supply to caries, starting cautiously in 1938⁴⁵ and culminating in the classical paper⁴⁶ correlating the examinations of some 7,000 children in 21 localities. The incidence of caries and of fluorosis in relation

to the fluoride concentration showed smooth curves, decreasing in the case of caries and increasing with fluorosis. The reduction of caries was nearly maximal at the level where fluorosis started to increase, and it was concluded that this level of 1 p.p.m. fluoride would be optimal for dental health. A number of other investigations have shown a rather similar relationship^{7, 60, 141, 142}, but in no case was there a sufficient number of points to specify an optimum concentration with any accuracy. This state of affairs is unfortunate, but in view of the small number of water supplies containing sufficient fluoride, and their widely scattered nature, is probably inevitable. An attempt has been made to overcome this defect by calculation from the mean annual temperature⁶⁶.

Numerous areas now have fluoride added to their water supply, and in several there have been detailed dental examinations over a sufficient period to demonstrate a marked effect on caries. The earliest studies were surveyed by a mission from Britain and their report⁵⁹ led to the fluoridation of water, in Anglesey and

Watford²⁸ and in Kilmarnock (see Appendix A).

The results of the surveys in these areas, which include some work carried out during the present investigation have been set out in an official publication³.

The initiation of fluoridation schemes has provoked violent opposition from a few individuals; many of the assertions made are quite unwarranted and many statements made in good faith have been used to support a case which was not within the ambit of the authors' consideration. In these circumstances, true discussion has been stifled since any criticisms of fluoridation are immediately taken as condemnation. Nevertheless, it must be said that fluoridation is not as effective as it might be, that it must be directed at a large number of people who receive no benefit, and that the control of the dosage to the individual, as distinct from the community, is probably rather imprecise, in spite of the narrow margin between toxic and therapeutic doses. There is clearly considerable scope for increasing both the efficiency of administration, and the effectiveness of the substances administered.

Both these aims require a knowledge of the metabolism of fluoride, and of its mode of action on caries so that factors which affect these can be examined and assessed.

It is against this background of empirical success and theoretical ignorance that the present investigation was started and the results represent little more than an examination of the directions in which progress might be achieved.

Ultimately, these processes must be described in terms of the processes occurring in the early dental cavity, and one approach must consider the chemistry of enamel, the chemistry of the enamel-fluoride interaction, and the chemistry of enamel dissolution: this is the approach from molecular properties. Molecular processes are, however, influenced by macroscopic features, so that another approach must consider the development of the carious cavity and the factors which influence it.

Thus, the work described here begins and ends in the carious cavity, and although the topics considered in between are in some cases rather distantly related to caries, the primary object is to lay the basis for an integration of

the two approaches described.

CHAPTER 2

THE PROBLEM

Dental caries is a disease which is almost universal in industrialised countries. It has also been increasing in severity in recent years: in the interval 1955 - 1963 caries in children in Ayr has almost doubled. This is a rather general statement because the exact ratio depends to a considerable extent on the group chosen and the method of measurement.

The first problem is therefore the measurement of caries. An international committee^a has recommended that caries should be reported as (1) prevalence or percentage of individuals with caries; (2) intensity, or total amount of caries per individual and (3) incidence or change in intensity per year. Prevalence is of limited practical importance in this country since the figures are almost always close to 100%. The intensity is measured in terms of the DMF index, which is the total number of decayed, missing or filled teeth per individual (the deciduous teeth are assessed separately and

designated by a df or dmf index). This index is therefore a cumulative index with respect to age. In fact in some areas the DMF is roughly a linear function of age (e.g. Appendix A) but this does not apply in all cases. The DMF has the advantages of simplicity, full use of the available scale, and effective demonstration of changes. The disadvantages are a lack of a linear scale (i.e. there is no justification for saying that DMF 2 represents twice as much caries as DMF 1, unless a circular definition of amount of caries is used), the obscuring of differential effects on different teeth, the dependence on the time of eruption of individual teeth and the large standard deviation, although the last is probably a result of the same effects which produce the useful discrimination.

To illustrate the practical effects of these considerations, the results of a survey in 1957 of the teeth of 297 Edinburgh children of 14 years of age (carried out in collaboration with Dr. J.N. Mansbridge) will be quoted. The mean DMF was 8.75 with a standard deviation of ± 3.80 . In order to detect a difference of 10% between two groups (which may be taken as the limit of

practical importance) it is therefore necessary to examine at least 76 individuals for a 5% level of significance. Furthermore with this size of group a sex difference has to be taken into account¹²⁴~~124~~. In the survey quoted the mean DMF for boys was 7.86 and for girls 9.75; the difference between these means would be significant at the 5% level in a combined group of 17 individuals. The age group chosen exaggerates this difference, but in general the difference between boys and girls is likely to be significant. Thus in the measurement of caries the subjects must be divided by age and sex and each group will require between 50 and 100 individuals, and the experiment is correspondingly time-consuming. A mathematical relation eliminating the effect of age would clearly be useful, and an approach to this is described in Appendix F.

The number of teeth about which there is doubt in the diagnosis for caries is small in relation to the total, so that the major part of the standard deviation of the DMF is due to biological variation. It appears therefore more useful at this stage to attempt to divide

samples into groups in such a way as to reduce the standard deviation, rather than to refine the methods of examination. For this purpose various types of test for caries activity are required, but those suggested so far are not particularly satisfactory. Correlations have been established between caries and bacterial counts⁴³, flow of saliva¹⁵², acid formation⁵¹, and carbohydrate consumption⁷⁶, but only in the last case is the relation other than weak. It would seem that tests should be devised which are more directly related to processes on the tooth surface. However, no test can be expected to measure the overall production of caries and if several factors influence the incidence of caries then a good correlation can only be obtained from simultaneous tests.

Effect of fluoride on caries

The official report³, which includes the results of studies in Kilmarnock, has confirmed the conclusions of similar American investigations at least as far as deciduous teeth are concerned,

that the addition of 1 p.p.m. fluoride to the drinking water produces a substantial decrease in the caries incidence. Further analysis of this work is not at present available for publication.

It has been found¹² that the reduction in carious surfaces is proportionately much greater in incisor teeth, which decay principally on the interproximal surfaces, than in molars (in which decay is principally occlusal). This again emphasises the difficulty of using a non-linear scale, since incisor cavities appear much more slowly than molar cavities. To make a comparison at the same age which is the normal procedure is to compare a group of molars in which most are carious already, and have been for sometime, with a group of incisors which have only just begun to decay. The comparison could only be fair if the incidence of caries in these teeth was constant with age, which it is not. More difficult is the problem of how to make a fair comparison: clearly one must first relate the caries incidence to both the age of the child and the age of the tooth. An attempt to do this has been made in ~~Chapter 6~~^{Appendix F}, although the results so far have been useful more as a qualitative

picture of caries than for quantitative assessments.

The age of a child at the start of fluoridation has a marked effect on the extent of the decrease in caries ^{10, 72}~~10, 72~~. The effect is in fact related to the age of the teeth, rather than the child, and for maximum effect the fluoride must have been consumed during the early stages of development. A slight effect has been reported on erupted teeth ⁸³~~83~~. These observations lead to the conclusion that the fluoride is effective by virtue of its incorporation into the teeth during development.

The direct application of fluoride to teeth after eruption has also been effective in decreasing caries, either as solutions of sodium fluoride ¹⁷⁸~~178~~ or stannous fluoride ⁶⁹~~69~~ or as toothpaste ¹³³~~133~~. These procedures have a limited period of effectiveness and must be repeated at appropriate intervals: no study has been carried on sufficiently long for an adequate comparison with water fluoridation, and some workers have had difficulty in reproducing the results ¹⁰⁰~~100~~, so that the relative effectiveness of the procedure is not clear. It should also be pointed out

that concentrated solutions are used, and the reaction, initially at least, is different.

Mechanism of fluoride action

If the action of fluoride occurs through incorporation into the enamel, it follows that there should be a raised concentration of fluoride demonstrable in enamel. This can readily be shown after topical application of fluoride, as might be expected¹⁷⁷; in an area (South Shields) where the drinking water contained approximately 1 p.p.m. fluoride it was found⁹² that the fluoride content of enamel increased from 90 p.p.m. at age 10 years to 180 p.p.m. at 44 years whereas in Leeds with less than 0.5 p.p.m. fluoride in the water the fluoride content was 58 p.p.m. at 10 years and 108 p.p.m. at 33 years. However it is known that enamel has a very much higher fluoride concentration in the surface: one report⁹¹ showed that in teeth from individuals aged less than 20 years, the surface enamel contained 500 p.p.m. fluoride, compared to about 50 p.p.m. for the inner layers

in an area with 0.1 p.p.m. fluoride in the drinking water; in teeth from an area with 1 p.p.m. fluoride in the drinking water the corresponding levels were 890 p.p.m. in the surface and 130 p.p.m. in the interior.

It is possible to suppose that a twofold increase in fluoride could be responsible for a 50% decrease in caries; on the other hand, the fluoride levels in enamel are so low that some form of concentrating mechanism appears to be a distinct possibility.

Jenkins has found⁹³ that oral bacteria are inhibited by 6 p.p.m. fluoride at pH 5 and has suggested that the fluoride is concentrated in the plaque on the surface of the tooth and released under acid conditions to inhibit the bacteria. It seems doubtful, however, whether a sufficient concentration of fluoride could be produced in the presence of salivary calcium without precipitating calcium fluoride.

A more likely concentrating mechanism derives from the fact that fluoride reacts with enamel (as discussed below), so that when enamel dissolves, the fluoride will react with the exposed enamel, producing an accumulation in the

area of solution. When enamel is dissolved in acid, little fluoride can be detected in the solution⁹⁰; in fact, the fluoride content of the enamel increases, although the solubility appears to be affected by other factors than fluoride⁹⁰. Carious enamel is known to contain more fluoride than the corresponding non-carious enamel¹¹⁰ and to take up fluoride preferentially¹³⁵.

Chemical properties of hard tissues

The first experimental investigation of fluoride uptake by teeth¹²⁵ was concerned with whole teeth. It was shown that decalcified teeth took up little fluoride from 0.7% sodium fluoride, and that carious teeth took up more than sound teeth. Since a tooth contains three different hard tissues, which are themselves structurally heterogeneous, it is not easy to specify precisely the nature of the tooth material used in an experiment; bone, on the other hand, is, at least superficially, more homogeneous, but a considerable amount of work has been done on calcium phosphates precipitated

in vitro, the composition of which can be controlled. In spite of the differences between these tissues, the inorganic component of each is a form of apatite, since the X-ray diffraction diagrams are similar³⁴.

The crystal structure of fluorapatite ($\text{Ca}_5(\text{PO}_4)_3\text{F}$) is known with a high degree of precision¹⁵, and hydroxyapatite gives a very similar diffraction pattern. Unfortunately it is impossible at present to grow large crystals of hydroxyapatite, so that the atomic co-ordinates are not known so accurately. Furthermore the atomic Ca/P ratio can vary from 1.3 to 1.7 in 'hydroxyapatite'¹⁴⁴ and in bone without affecting the diffraction pattern. Surface effects account for some of this variability³⁵; another possibility is the substitution of hydrogen ions for calcium in the apatite lattice¹⁴⁴, which is supported by the formation of pyrophosphate on heating⁸⁹. Hydrogen ions cannot be located directly by X-ray diffraction, but more recent work³⁰ has suggested that hydroxyapatite and octocalcium phosphate $\text{Ca}_8\text{H}_2(\text{PO}_4)_6 \cdot 5\text{H}_2\text{O}$ (which also gives an apatite diffraction pattern) can form mixed crystals.

The crystal size in apatites precipitated in vitro depends on the conditions and is an important property of the material, since the surface area is always large. The crystal size in bone is more constant: from X-ray diffraction measurement³⁶ the length is 600 - 700 Å. (corresponding to the collagen banding at 640 Å intervals) and the diameter is 50 Å. Electron microscopy⁵⁶ has, however, suggested a length of 200 Å. Enamel crystals are about 1600 x 400 x 170 Å¹⁵⁰ and the surface area is 1.8 sq.m./gm. compared with 100 sq.m./gm. for glycol-ashed bone¹⁸⁸.

Access to the crystal surface is determined by the density of the organic matter between the crystals, which binds the structure together. In bone, dentine and cementum the organic fraction represents about 20% mostly in the form of collagen fibres. In enamel there is only 0.5% w/w protein; the only other organic component present in comparable amounts is 0.1% citrate¹⁷¹. If all the organic matter was evenly distributed on the crystal surfaces the thickness would be about 30 Å. indicating a crystal separation of 60 Å. In fact the organic matter is not evenly

distributed and there is a variable amount of water to accommodate (2-4%). Most of the organic matter occurs in certain special structures visible under the optical microscope (lamellae, tufts and prism sheaths)¹⁶⁸. There is clearly also a fine framework of organic matter surrounding the crystals, and visible under the electron microscope¹⁰⁹, but owing to difficulties of fixation, the thickness has not been measured.

It has clearly been shown that water will flow through enamel of extracted teeth, under external forces¹¹, and can be forced out, presumably by cellular action, from teeth immediately after extraction²¹. An indication of the size of the pores has been obtained from investigations of the effect of liquids of different refractive indices on the retardation of polarised light⁴². Intact enamel was penetrated readily by methanol, but not by any higher alcohols, suggesting that the pores are about 5 Å. across.

The solubility of calcium phosphates has attracted much attention, partly because of the complex nature of the standard solubility product,

but more particularly because the solubility varies with solid/solution ratio¹⁰⁵~~105~~. As this ratio was increased, the calcium in solution increased, but the phosphate rose to a maximum and then decreased. This is clearly a surface effect, but the explanation suggested by Neuman¹³⁹~~139~~ has been challenged¹⁵¹~~151~~ and further work is still needed.

Mechanism of fluoride incorporation

In a comparative survey¹⁶⁰~~160~~ of the fluorine content of teeth from India and U.S.A., the Indian teeth contained 5 - 10 times as much fluorine, although there was little difference in the fluoride in the water supply. Some of this fluoride might have come from the food, or from the higher water intake in a hot climate, but this is not sufficient to account for the marked difference. A possible explanation is that something else is required in the diet to achieve a high fluoride level, that is there may be a natural, possibly enzymic mechanism with this function.

A difference in the prevalence of caries in two Hungarian towns¹³⁷ was explained on the basis of the difference in the molybdenum content of the water, and this was confirmed by a similar investigation in New Zealand⁸⁴. The amount of fluorine in the femur of rats on a diet containing 0.08 µg./g. fluorine was almost doubled by administering 0.25 mg. molybdenum daily¹⁷⁴, and molybdenum in the drinking water had a synergistic effect with fluoride on caries in rats¹⁷⁵. Molybdenum deficiency is not common however, and can hardly be expected to be more than a sporadically important factor in human caries.

Following an intravenous injection into rats of 0.1 µg. fluoride labelled with ¹⁸F, some 50% of the radioactivity was recovered in the skeleton, 30% was excreted in the urine and the remainder was distributed through the soft tissues. The teeth contained about 1.5% of the dose and the half-time of disappearance of fluoride from blood after 30 minutes was about 15 minutes¹⁸². The metabolism of fluoride is relatively rapid, and the major part is taken up in areas where active calcification is

proceeding. Excretion of accumulated fluoride continues for several years when the fluoride intake is reduced¹⁰⁶, but this is probably due to replacement (remodelling) of bone rather than loss from existing bone.

Ingested fluoride is thus normally incorporated into teeth from tissue fluid, which is assumed to have the same fluoride level as blood, currently accepted to be in the region of 0.1 - 0.15 p.p.m.¹⁶³ although Hall⁷⁹ has recently claimed lower values, too small to measure. On the other hand topical applications of fluoride are always carried out with concentrated solutions and the primary product of the reaction is calcium fluoride¹⁵⁸, together with stannous phosphate if stannous fluoride is used³⁹. There is no doubt, however, that calcium fluoride is slightly soluble in saliva, and any precipitate formed on a tooth in the mouth will eventually dissolve away. In the process the tooth will be bathed in a low concentration of fluoride ions over a considerable period. Whichever method of fluoride incorporation is being considered, it is of interest to enquire

into the process of fluoride uptake at low concentrations.

The difference in the reactions at high and low fluoride concentrations was discovered during the War in Germany⁶⁸. In concentrated solutions CaF_2 was formed and phosphate liberated into the solution; in dilute solutions, little phosphate was liberated, but the solution became alkaline, indicating replacement of hydroxyl in hydroxyapatite by fluoride. Calcium fluoride is formed when its solubility product is exceeded¹⁰³. However, since the reaction was started in a fluoride solution containing no calcium, the precipitation of calcium fluoride would depend on the rate at which calcium dissolved compared to the rate at which fluoride was taken up. Consequently, the amount of calcium fluoride depended on the solid/solution ratio¹¹³.

The equilibrium between hydroxyapatite and fluoride takes so long to be established that it is doubtful whether it has ever been achieved. Elephant and mastodon molars from the Lower Pleistocene (c. 10^6 years) showed an $\text{F}/\text{P}_2\text{O}_5$ ratio of $0.064 - 0.083$ ¹⁴².

(theoretical maximum for fluorapatite 0.089), and there is no evidence that the reaction had stopped. Allowing for the possibly low concentration of fluoride in the ground water surrounding the teeth, and the effects of several Ice Ages, it can be stated that equilibrium is unlikely to be attained in a laboratory experiment. One attempt has actually been made¹³⁸ in which a solution of 2 p.p.m. fluoride was passed through a column of glycol-ashed bone until the fluoride content of the effluent was not detectably different from the solution flowing in. This took 80 days, and the final fluoride content of the bone was 1%. (The fluoride content of fluorapatite is 4%).

In spite of the lack of equilibrium there is no doubt that the reaction can be reversed. Smith and Davey¹⁶⁵ showed that bone powder used for removing excess fluoride from water could be regenerated with alkali, and McCann¹¹⁵ showed that the longer enamel had been in contact with fluoride, the less was the proportion of fluoride removed in a given time by N potassium hydroxide. Further, the uptake of fluoride has been shown to

depend on the pH over a limited range¹³⁸. No investigation has been found on the effect of hydroxyl ions on pure fluorapatite, although this would seem to be a useful line.

Many investigators have been content to examine the reaction after a single time interval: Leach¹⁰³ chose six hours and stated 'little farther uptake occurs'; McCann¹¹⁴ used three to four weeks without comment; Ericsson⁵³ used thirty minutes, again without comment.

Studies on the time course of the reaction have also produced very varied results: Adler¹⁷⁶ concluded that the reaction was complete in one hour (at which stage the enamel contained 0.038% fluorine); the Indiana workers⁸² found a steady increase in fluoride uptake over 25 hours, while McCann¹¹⁵ found that the uptake was higher after one week than after one day and higher still after 8 weeks. These variations are no doubt partly due to the different conditions used, but also to the problem of obtaining results sufficiently accurate to measure slow rates of reaction. In no case has a quantitative analysis of enamel uptake been attempted.

More consistent results have been obtained with radioactive fluorine, although the short half-life limits experiments to about 10 hours. Hardwick⁶² published a curve showing the uptake of ^{18}F by an intact enamel surface over a twelve hour period without any comment on its significance. If this curve is replotted against the square root of time an almost straight line is obtained. Further work⁸⁰ showed that under similar conditions the mean depth of penetration of the fluoride was about 100 microns, so that the amount of fluoride which could have passed through the enamel was negligible. These results strongly suggest that the reaction is diffusion-limited. The time course of the fluoride reaction with hydroxyapatite also follows the same curve⁷⁵; the 'saturation value' reached after 12 hours is related to the surface area, and there is an initial rapid uptake, presumably due to adsorption. This clearly divides the reaction on a small crystal into three stages: (1) adsorption; (2) diffusion into the crystal surface; (3) diffusion into the crystal interior.

The third stage must be much slower than the second, but it is possible that the diffusion constant decreases steadily towards the crystal interior. These stages were postulated by Neuman¹⁸⁴ for the uptake of radioactive phosphate by hydroxyapatite, but the process was analysed in terms of sequential exponential reactions such as occur in radioactive decay series. It should be noted that only one set of points is given to justify this analysis, and these points, presumably the best values obtained, could just as well be fitted with a smooth curve. Further, this type of analysis is based on the assumption that each reaction proceeds simultaneously, although the model proposed is sequential; in addition if a reaction in a sequence does not proceed to completion the rate of the succeeding reaction depends on the position of equilibrium, which in turn depends on the measured variable, the amount of material remaining in the solution.

As far as enamel is concerned, one has to consider separately (1) the reaction of the exposed surface; (2) the diffusion through the pores (3) reaction with the internal crystal

surfaces. In Hardwick's experiment⁶², the first is negligible, and the second is probably rate-limiting since if the third were rate-limiting the reacting surface area would be growing continuously and a simple diffusion relation could not be expected. In experiments with powdered enamel on the other hand the diffusion through the pores is likely to be complete in a few hours (judging from the rate of penetration⁸⁰), after which one is effectively studying the reaction of the entire accessible crystal surface.

Investigation of reaction

In order to study the reaction of fluoride with enamel one must have methods available for (1) isolating the enamel in a suitable state; (2) carrying out the reaction without interference; (3) measuring the fluoride; and (4) comparing different samples of enamel.

As far as the isolation of enamel is concerned, it is clearly preferable to use intact enamel, but the uptake of fluoride is so small

that only radioactive methods are suitable. The alternative is to use powdered enamel, which can be more homogeneous, but has the disadvantage of an exposed surface not normally present, and a greater risk of contamination (with dentine).

The two main sources of interference in this type of experiment are loss of fluoride by adsorption on glass¹⁷⁰~~183~~ and growth of micro-organisms. The first requires that glass should be avoided as far as possible, and that sintered glass should not be used at all. The second requires the addition of a suitable chemical, since sterilising enamel without altering its protein, and maintaining sterility during repeated sampling, present considerable difficulties. Levinskas¹⁰⁵~~106~~ found chloroform satisfactory in studying the solubility equilibrium of hydroxyapatite.

Measurement of fluoride

It has already been pointed out that in the reaction between fluoride and developing teeth the fluoride is probably present at a concentration

in the region of 0.1 p.p.m., and the same is probably true of the reaction between salivary fluoride and erupted teeth⁶⁷. It is therefore of primary interest to investigate the reaction in vitro at concentrations of this order, and a method for the accurate measurements at this level is required. This is possible by standard methods if large volumes are available, as, for example, in water analysis, but when the sample is restricted to 1 ml., no available method is both sufficiently sensitive and sufficiently accurate.

The standard method of fluorine analysis involves ashing (if organic matter is present) steam distillation to remove fluoride from interfering metals and anions, and estimation by competition with a dye for complexing a metal¹²⁷. The limiting factor in this procedure is the distillation³⁸. The introduction of diffusion of hydrogen fluoride from concentrated perchloric acid¹⁶² when polythene bottles became available has greatly reduced the blank value and the volume of the liquid in which the fluoride is collected. More recently the use of a polypropylene Conway unit has been suggested¹⁶⁴.

Several metals and numerous dyes have been suggested for the final analysis⁵⁰, with little to choose between them. In all cases the estimation depends on the decrease in colour of the metal-dye complex on adding fluoride. The increase in free dye could be measured but the extinction is usually smaller. A considerable advance came with the synthesis of alizarin complexan¹⁶, a combination in one molecule of alizarin (a popular dye for fluoride measurement) and a chelating agent, which forms a red complex with several rare earth elements and a blue ternary complex with the rare earth and fluoride¹⁷. It is now accepted that the lanthanum complex gives the best results¹⁸. The blank value is rather high, but can be greatly reduced by extraction with 3% hydroxylamine hydrochloride in isobutanol⁷⁹, and an increase of optical density of 0.1 in a 1 cm. cell is then obtained with 0.4 μ g. fluoride. This procedure is more specific than other methods, but phosphate causes severe interference, as with almost all other methods using a metal.

Other methods of estimating fluoride have

given no better results. Of electrical methods, polarography has appeared the most promising¹²⁰~~120~~ but is still subject to phosphate interference, since a metal plays an essential part. This is unfortunate, because if fluoride could be estimated in the presence of phosphate, fluoride uptake by enamel could be carried out without separating the fluoride, and with a consequent reduction in the possibilities of error. The only sensitive method which is said to be free of phosphate interference⁵⁰~~50~~ is the inhibition of esterase⁹~~9~~, but it has never been investigated thoroughly nor was it adequately confirmed until fifty years later¹⁰⁷~~107~~.

Plan of Study

The primary aim of this investigation has been to develop a method for the analysis of fluoride which met the requirements discussed above. In the first instance a polarographic method was chosen, and used to evaluate the diffusion separation required concomitantly: the results are described in the next Chapter.

In Chapter 4 the purification of esterase from liver is described, together with an investigation of the kinetics of the enzyme and the development of a suitable procedure for using it.

The preparation of enamel in a suitable form was also investigated and described in Chapter 5 together with some measurements of its reaction with fluoride.

Chapter 6 contains a general discussion, Chapter 7 a summary of the whole work, and Chapter 8 the bibliography in alphabetical order. In Chapter 9 are included reprints of all the papers related to the subject of this investigation published by the author. Except where continuity makes it essential, no material from these papers has been included in the other chapters, but references to Chapter 9 appear at appropriate points in the text.

CHAPTER 3

POLAROGRAPHIC STUDIES

The original technique of polarography developed by Heyrovsky, consisted of the measurement of the current flowing through a cell composed of a reversible anode, a conducting electrolyte and a mercury drop cathode when the applied voltage was varied. A relatively simple electrical circuit can be used for manual recording of the current.

The component which gives the system its useful properties is the mercury electrode. In its usual form, a fine glass capillary is broken off squarely and placed with the broken end in the cell, and the other end connected to a reservoir of mercury. The drops of mercury which form at the lower end of the capillary are practically spherical; the rate of formation can be changed by using different capillaries, and by altering the reservoir height. In this type of system the mean current over the life of the drop is usually recorded.

When the cell contains only the base

electrolyte, a small residual current is found, which rises steeply when the voltage is increased to the point where discharge of hydrogen ions occurs (i.e. electrolysis). If a substance which is more easily reduced than hydrogen is present, a current is produced due to the reduction process: this current is limited by the rate of diffusion of the reducible material up to the mercury drop, and because the latter is spherical and continually replaced, this diffusion current is proportional to the concentration of the reducible substance (providing the reduction is reversible). There is a characteristic voltage (the half-wave potential) for each substance below which little reduction occurs and above which the diffusion current becomes established.

When the reduction is not reversible, it is usual to carry out an amperometric titration, in which the current is measured at constant voltage when the reducible material is removed by the addition of suitable reagent. The reverse process can also be used: fluoride has been determined by measuring the progressive change in the diffusion current of thorium

nitrate when titrated with the fluoride solution¹⁰²~~102~~. The method was suitable for 3 - 30 μ g. fluoride.

It is possible to measure small currents very accurately and polarography is theoretically capable of a very high sensitivity. Attempts have been made to improve the original polarograph by using solid electrodes. These are inherently less stable than the mercury electrode, but Shoemaker¹⁶¹~~161~~ claimed to measure 10 μ g. fluoride with an error of less than 3% with a rotated platinum electrode, and 1 - 2 μ g. with 7 - 12% error.

More successful have been developments in the use of a varying voltage and more refined means of analysing the current¹²⁹~~129~~. These include derivative polarography, oscillographic polarography with an alternating current imposed on the direct current, and square-wave polarography⁵⁷~~57~~. Another variant attracted attention by the publication of a sensitive method for the determination¹²¹~~121~~ of fluoride.

This instrument, known as a cathode-ray polarograph, is designed to apply to a single mercury drop a steadily increasing voltage, and

to display on an oscillograph screen a plot of current flow against applied voltage⁴⁴. A capillary is used which gives a drop time in the region of seven seconds, and the voltage sweep is applied during the last two seconds of the life of the drop in order to minimise changes in surface area.

The advantage of this instrument is that when the applied voltage reaches the half-wave potential of the substance being examined, all the molecules in the region of the electrode are available for reduction. The oscillograph therefore shows a marked peak in the region of the half-wave potential followed by a fall to the diffusion current level as the steady state is established. Some illustrations of these peaks are shown in Appendix D.

The method for measurement of fluoride is necessarily indirect since fluoride does not give a polarographic wave. It is found that the peak given by certain o-hydroxy azo-dyes (due to reduction of the azo-group) is decreased in height by the presence of aluminium and that a second peak is formed. Fluoride decreases the height of this second peak by complexing with the aluminium¹⁸⁷. The procedure described¹²¹ for

the determination of fluoride by this means was examined to assess its usefulness.

0.5 - 2.5 μ g. fluoride in a few ml. water was placed in a 25 ml. flask and 5 ml. freshly mixed reagent was added. The reagent was made up from stock solutions to contain 0.6 μ g./ml. Al (from potassium alum), 0.4 N ammonium acetate pH 3.6, 0.02 N ammonium chloride (to react with the Ag/AgCl anode) and 0.01% Solochrome Violet RS. (Proteose peptone was recommended¹²¹ as a maximum suppressor, but this was omitted since no maxima were ever encountered with this solution). The ammonium acetate was adjusted to pH 3.66 with concentrated perchloric acid and shaken with 1 ml. 1% acetylacetone to extract impurities. The acetylacetone was removed by extracting five times with 25 ml. portions of chloroform, and the solution was heated to remove the chloroform, resulting in a small rise in pH.

All the flasks for a particular series were collected together, made up to volume and heated together for 30 minutes at 65 - 75°C. The following day the solutions were placed in

the polarograph cells in a water-bath at 25°C. and oxygen-free nitrogen was bubbled through the cells for 20 min. The mercury cathode and a silver wire which formed the Ag/AgCl anode were placed in each solution in turn and the shape of the polarographic waves was observed on the screen of the cathode-ray polarograph over the full voltage range.

Two waves were obtained, a large one at about -0.4 V. and a small wave at -0.55 V. Alteration of the concentrations of aluminium and dye showed that the height of the first wave was primarily dependent on the dye concentration and the height of the second wave varied with both aluminium and dye concentrations. Fluoride decreased the height of the second wave, but the results were somewhat variable. While testing possible causes for this, it was noticed that the blank value varied with positions of the flask in the series and a check of this showed a systematic error. All the flasks contained reagent, but no fluoride and were treated as far as possible in an identical manner, in the order in which they were lettered. The wave heights obtained were:

[P.T.O.]

Flask	Wave height μ amp.
A	0.396
B	0.406
C	0.426
E	0.428
F	0.462
G	0.454
H	0.486
I	0.492
K	0.52
L	0.55

The source of this systematic error remained obscure until it was noticed that late in the evening the setting sun fell on the flasks which had been left for polarography the following morning. The previous experiment was therefore repeated, except that the flasks were placed in a cupboard immediately after heating. The result for the same flasks was:

Mean	0.7035 μ amp.
Standard deviation	± 0.0075
Coefficient of variation	1.1%

This procedure was therefore followed in all further work. The figures also give an indication of the accuracy of the instrument used.

The effect of fluoride was then re-examined:

F added μg.	Wave height μamp.	Depression	
		μamp.	μamp./μg.
0	0.725		
0.5	0.660	0.065	0.130
1.0	0.615	0.110	0.110
1.5	0.570	0.155	0.103
2.0	0.530	0.195	0.098
2.5	0.485	0.240	0.096
3.0	0.445	0.280	0.093
4.0	0.375	0.350	0.088
5.0	0.320	0.405	0.081

It was stated by MacNulty et al.¹²¹ that this calibration was linear up to 2.5 μg.; this result has not been confirmed, but the reproducibility appeared to be satisfactory. In later work it was apparent that a calibration curve drawn by eye had little advantage over a straight line since the random error in the former was of the same order as the systematic error of the latter.

Since 3 μg. Al (contained in each flask) corresponds to 2.1 μg. F, it may be noted that formation of AlF_3 would produce a depression of $0.725/(3 \times 2.1) = 0.115$ μamp./μg. F. Under the conditions of high dilution, a series of Al/F complexes are probably present but this figure suggests that the uncharged complex predominates.

In order to make full use of an instrument

T A B L E I

Reproducibility of polarograph measurements

Added $\mu\text{g. F}$	Reading $\mu\text{amp.}$	Expected $\mu\text{g. F}$	Difference $\mu\text{g. F}$	Mean expected $\mu\text{g. F}$
0	1.200	-0.0261	-0.0261	+0.008
0	1.195	+0.0107	+0.0107	
0.5	1.135	0.4524	-0.0476	0.502
0.5	1.125	0.5261	+0.0261	
0.5	1.125	0.5261	+0.0261	
1.0	1.065	0.9678	-0.0322	1.066
1.0	1.045	1.1150	+0.1150	
1.0	1.045	1.1150	+0.1150	
2.0	0.935	1.9249	-0.0751	1.980
2.0	0.920	2.0353	+0.0353	
Standard deviation			± 0.0623 (n = 10)	

From regression of amount of fluoride on reading:

		Standard error
Slope	-7.362	
Intercept (for no F added)	1.196 $\mu\text{amp.}$	± 0.030
Mean F	0.850 $\mu\text{g.}$	± 0.018

Coefficient of variation of mean F = 6.1%

Coefficient of variation of mean reading = 0.65%

[To face page 47

it is important that the largest quantity measured should be adjusted to produce a maximum deflection, at least when there is a linear scale such as the cathode ray polarograph possesses. The full scale deflection on the range considered was 1.25 μ amp. and the amount of aluminium was increased in order to bring the blank value nearer to this point. An amount of 5 μ g. aluminium per flask was found convenient, although the actual height of the wave varied somewhat between experiments. This is attributed to the particular conditions of overnight incubation and emphasises the necessity for running standards for every measurement, and ensuring that all flasks receive precisely similar treatment.

The reproducibility was then checked using this larger quantity of aluminium. The results are shown in Table I and Fig. 1.

In order to obtain an estimate of the variability, the regression line for the calibration graph was calculated. Since the primary interest is in the analytical possibilities, the amounts of fluoride were regressed on the readings obtained, so that the

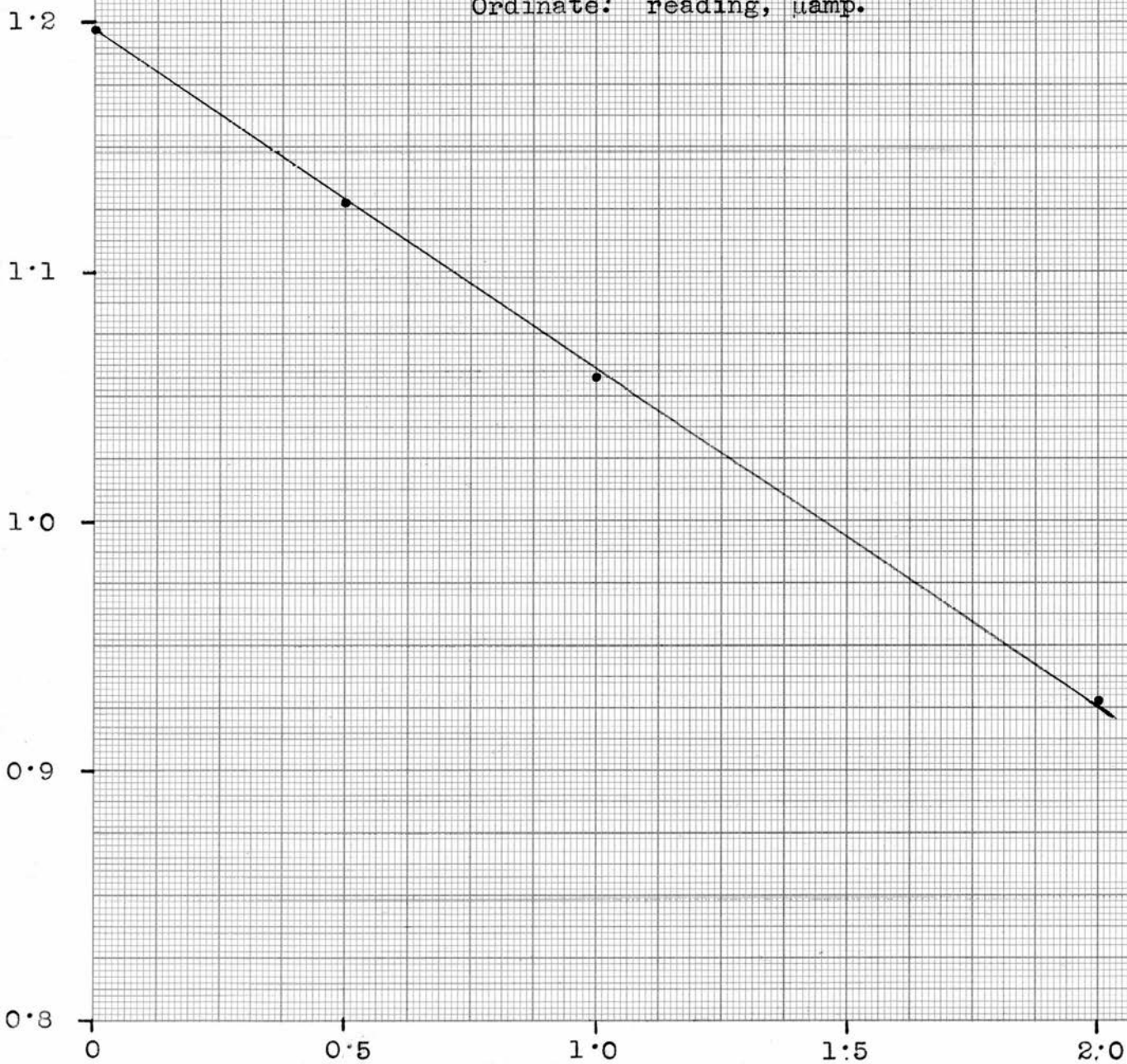
Fig. 1

Calibration curve for polarograph

Mean values from Table I plotted

Abscissa: fluoride, $\mu\text{g.}$

Ordinate: reading, $\mu\text{amp.}$



parameters obtained are the best available for estimating unknown fluoride concentrations from observed readings. Other calibration graphs to be discussed later have been treated in a similar manner to facilitate comparison.

The graph in Fig. 1 is clearly linear within the experimental error. The intercept (Table I) is less than one standard error from the observed values, and there is no systematic shift in the differences between the observed and calculated values for the fluoride concentration.

The standard deviation for this particular range is $\pm 0.062 \mu\text{g.}$ The originators of the method quoted a reproducibility of $\pm 10\%$ for the range up to $10 \mu\text{g.}^{23}$, without specifying how this was calculated. The coefficient of variation in the present series was 6.1% in a lower concentration range, which compares favourably with the original claim.

It may be noted, however, that when the coefficient of variation is calculated on the basis of the readings taken, it is almost ten times as small, and close to the minimum difference between readings. It is therefore probable that the main cause of variability is an instrumental

one; and although the instrument is satisfactory for direct readings, the transformation required to estimate amounts of fluoride magnifies this variability greatly. Nearly all methods for measuring low levels of fluoride are based on a decrement effect, and their accuracy is decreased correspondingly.

Separation of fluoride

Methods of fluoride analysis which make use of the complexing ability of metals are affected by interference from the presence of other metals, and of anions such as phosphate which complex the metals. The standard procedure for removing this interference is steam distillation of the fluoride as fluorosilic^{ic} acid. The sample is mixed with concentrated perchloric (or sometimes sulphuric) acid and a little sand, steam is passed through while the acid is held close to 138°C., and about 150 ml. distillate is collected in dilute alkali. This procedure was tested, but satisfactory results could not be obtained when less than 10 µg. fluoride was used. Part

of the difficulty with this method lies in the extreme dilution produced, but there is also a blank value approximating 1 μ g. fluoride.

The construction of a microstill was considered, but a method which appeared more convenient had been published a short time before and it was decided to examine this procedure¹⁶²~~178~~. The original description specified placing 1 ml. sample in a 2 oz. polythene bottle and freezing. The bottle was then transferred to a Dewar flask, and 2 ml. cold perchloric acid was added by means of an all glass syringe with a three inch fixed glass needle. A receiving strip was made up from a sheet of polythene roughened on one side; a small amount of sodium hydroxide solution was placed on the roughened area and dried in a desiccator. One of these strips was placed in the bottle immediately after the acid and the cap was screwed on. The bottle was then incubated at 50°C. for 20 hours to volatilise the hydrogen fluoride, allowing it to be absorbed by the sodium hydroxide. The strip was removed, and the lower end was cut off; the strip was placed in a tube of water and centrifuged to remove air bubbles, and then shaken on a mechanical shaker.

With this procedure, the originators obtained 99% recovery after 20 hours, but lower values at shorter times, using 20 μg . fluoride. The blank was stated to be 0.012 μg . with a standard deviation of ± 0.013 , so that there appeared to be a good chance of satisfactory results with much lower levels of fluoride.

Certain modifications in this procedure were made in carrying out preliminary trials. It appeared unlikely that any fluoride could be lost in the few seconds between adding the acid and putting on the cap, when complete diffusion required many hours and an elevated temperature. Since the equipment necessary for cooling was not readily available, this part of the procedure was omitted initially, with the possibility of testing it later if good recoveries could not be obtained. Hall⁷⁸₄₀ confirmed that freezing was unnecessary.

The polythene strips were suspended from grooves cut in the neck of the bottle, which avoided the possibility of perchloric acid creeping up the strip. The preparation of these strips occupied a considerable proportion of the time required for an analysis, and at the



suggestion of Dr. J.K. Grant they were replaced by pieces of platinum gauze, which were suspended from a polythene cross-piece. The sodium hydroxide solution was spread on the gauze with the pipette and dried in an oven just before use.

The procedure adopted for testing was therefore as follows:

Polythene bottles of 75 ml. capacity were slotted to take pieces of polythene sheet, on which could be hung pieces of 60 mesh platinum gauze. The gauze was covered with 0.05 ml. N NaOH and placed in an oven to dry. 1 ml. of sample solution was placed in a bottle and a dried gauze was hung in position. 2 ml. 72% HClO_4 was added quickly by means of an all-glass syringe with a fixed 3'' capillary needle, and the lid was immediately screwed down. The bottles were swirled gently to complete the mixing and incubated in an oven at 60°C. for 20 hours. The pieces of gauze were then placed in polythene tubes and covered with water. Methyl Red was added and just sufficient 0.1 N HClO_4 to give a permanent red colour. The solution was then washed into a volumetric flask containing the reagent, and

the fluoride was estimated polarographically as already described.

The initial results were somewhat erratic; an investigation of the effect of phosphate on diffusion illustrates the characteristics of the method: 0.5 ml. 0.1 M phosphate was added to two diffusion bottles together with 0.5 ml. 2.5 p.p.m. fluoride solution. In two other bottles water was added. The results in $\mu\text{amp.}$ were:

	Standard (not diffused)	Diffused
No F added	0.990	0.925
F alone	0.850	0.775 0.800
F + phosphate		0.795 0.870

The blank value is here equivalent to about 0.58 $\mu\text{g.}$ fluoride. The disagreement between the duplicates makes any firm conclusion about the effect of phosphate rather difficult.

The variation of the blank value was examined as a possible source of error. Five portions of distilled water were diffused, and 2 $\mu\text{g.}$ fluoride was added to two of the tubes in which the gauzes were eluted after diffusion.

The measurements in μ amps were:

	Fluoride added		Mean difference
	None	2 μ g.	
Not diffused	{ 0.940 0.955	{ 0.745 0.760	0.195
Water only diffused	{ 0.900 0.915 0.925	{ 0.705 0.710	0.205
Mean difference	0.034	0.045	

Analysis of variance gives:

Effect	Sum of square	n	variance
Fluoride	0.0862	1	0.0862
Diffusion	0.0000 0.00080	1	0.00080
Interaction	0.00263	3	0.00088
Replication	0.00554	3	0.00019

The interaction and diffusion variances are considerably larger than the replication variance but the ratios are not significant. It is probable that diffusion introduces a larger error, and although the effect is not significant the standard deviation due to diffusion alone is given as 0.025 compared with 0.014 for the actual measurement procedure. It appeared unlikely that variable blanks were affecting the results greatly.

At this stage, owing to the failure of the polarograph, it was necessary to use a higher level of fluoride which was measured by titration with thorium nitrate⁸⁸.

After elution of the gauze which had been diffused, the solution was neutralised using phenolphthalein as indicator and placed in a Nessler cylinder with 1 ml. 0.4 N monochloracetate buffer pH 2.9 and 1 ml. 0.03% alizarin sulphonate and titrated with 0.1 mM thorium nitrate to match a blank containing 0.3 ml. thorium solution.

An indication of the cause of the variability was obtained when the period of diffusion was altered. 20 µg. fluoride was placed in the diffusion bottle and the amounts found on the gauze after diffusion were:

Diffusion time (hr.)	16	41
No. of measurements	4	2
Mean fluoride found µg.	18.5	20.4
Standard deviation	±0.82	0

A more extensive check of the effect of diffusion time was therefore made. Using

20 μ g. fluoride the results were:

Diffusion time (hr.)	16.5	20	24	40	65	90
Recovery %	73	84	92	94	92	98

Using 10 μ g. fluoride:

Diffusion time (hr.)	45	70
Recovery %	94.4	101
Standard deviation	± 1.5	± 3.6
No. of bottles	8	8

The accuracy of the titration varied with the quality of daylight available. The reason for the long time necessary for completion of the diffusion has never been ascertained. The dimensions of the bottles used for diffusion were of the same order as those in the original description¹⁶² so that the surface area and depth of the liquid would be comparable. The difference might lie in the surface area of the sodium hydroxide absorbent. It may also be significant that quite recently it has been reported that with a polythene Conway diffusion cell, perchloric acid gives poor recoveries

compared with sulphuric acid⁷⁵.

With the repair of the polarograph a further check on the diffusion method was made at a lower level of fluoride. Blank values were first determined for all the diffusion bottles: the mean reading obtained for 13 bottles after 4 days incubation at 60°C. was 1.432 μ amp. with a standard deviation of ± 0.027 . This gives a coefficient of variation of 1.9%, which is higher than the value obtained for the polarograph itself (Table I). The variance ratio is not quite significant at the 0.1% level, so that the diffusion procedure clearly introduces an additional source of variation. In the same experiment the mean reading for three undiffused blanks was 1.475 μ amp. The difference between these two means was equivalent to 0.13 μ g. fluoride, and was less than two standard deviations. It was therefore concluded that values of the blank for individual bottles would not be sufficiently reproducible to make their use worthwhile, and a mean value was employed.

Next, 2.5 μ g. fluoride was diffused in the bottles in two experiments and the polarograph

readings were compared with the corresponding values without diffusion:

Fluoride added	none	2.5 μ g.	2.5 μ g.
Diffusion	no	no	yes

Experiment 1

No. of observations	3	3	12
Mean reading μ amp.	1.567	0.822	0.842
Standard deviation	± 0.005	± 0.005	± 0.010

Experiment 2

No. of observations	3	3	12
Mean reading μ amp.	1.523	0.802	0.801
Standard deviation	± 0.013	± 0.006	± 0.028

Assuming that the blank value determined in the previous experiment is applicable here, the results indicate that recovery of fluoride is not quite complete. The recoveries are respectively 92% and 95%. The ratio of the variances of the two sets of diffusion measurements is significant at the 0.1% levels although there was no difference apparent in the conditions of the two experiments sufficient to account for this result. The variance of the diffusion measurements in Experiment 1 does not differ significantly from the variance of the readings in Table I.

The standard deviations of the diffusion measurements in terms of fluoride are ± 0.034 $\mu\text{g.}$ and ± 0.097 $\mu\text{g.}$ respectively. These values cannot strictly be compared with Table I since the slope of the calibration graph has changed, but it seems probable that in Experiment 1 an exceptionally low value of the standard deviation has occurred by chance, and later calibration curves have usually shown a higher variation.

The coefficient of variation of the estimate of fluoride in Experiment 2 is 3.9%. This appears not unreasonable, but it must be observed that if 1 $\mu\text{g.}$ had been measured with the same standard deviation, the coefficient of variation would have been 10%, which is distinctly high.

It appeared that the variability introduced by diffusion was somewhat higher than that inherent in the polarographic measurement, but not sufficiently greater to be readily detected. Each set of measurements took one week for completion and it was felt that the amount of work involved in attempting to improve further the accuracy of the diffusion procedure would not be justified by the possible results.

The procedure was used as it stood in a

number of investigations, and some useful information was obtained, but on the whole the results were not encouraging and the possibility of devising a more suitable method for fluoride analysis was considered.

Polarography of arsonic acids

If one adds fluoride to a solution of a suitable metal complex, the fluoride forms a new complex with the metal, leaving free an amount of the original complexing agent proportional to the amount of fluoride. This is the basis of almost all methods of fluoride estimation, but in most cases the properties of the system make it necessary to measure the amount of metal complex, which decreases with added fluoride. The effect of a metal on the polarographic wave of a complexing agent depends on whether the electrons of the reducible group are involved in the bonding: if this is so, then the wave will be moved to a greater negative potential, and possibly to such an extent that no interference occurs with the wave of the uncomplexed substance

(although the rate of dissociation of the complex will also be a determining factor).

The metal chosen for examination was thorium, because it has the highest affinity for fluoride in a convenient pH range^{2.4}. Over a period, the effect of fluoride on a large number of thorium complexes was examined. Several interesting observations were made, and a study of the basis of these effects is described in Appendix D. As far as fluoride analysis was concerned, only two compounds were found to show the increase for which the search was made. One of these was Chrome Azurol S, already well known as a reagent in fluoride determination¹³⁰. Since almost all the dye in solution was precipitated by standing overnight with thorium it is probable that thorium decreases the polarographic wave by polymerisation. There was a relatively large blank value, but between pH 2 - 3.5 the wave height showed little change. The change produced by fluoride was however only $\pm 0.012 \mu\text{amp./}\mu\text{g.}$ (compared with $-0.14 \mu\text{amp./}\mu\text{g.}$ for Al/Solochrome Violet RS), which implied a considerable loss of sensitivity.

The other substance which showed promise was

o-nitrobenzenearsonic acid. The final method is described in Appendix E. The polarogram of nitrobenzenearsonic acid shows two main waves, one at about -0.25 V., the other at about -0.80 V. On addition of thorium the first wave is unaffected, and the second wave decreases in height; on adding fluoride also, the second wave increases in height, the first again being unaffected. This is a most useful effect, since the ratio of the heights of the two waves provides an internal comparison which eliminates possible variations in concentrations between samples, (of importance where the adjustment of pH in small samples is necessary) and increases the accuracy of measurement. The change in the second wave at the level of 10 $\mu\text{g.}$ fluoride was 0.33 $\mu\text{amp./}\mu\text{g.}$ At higher levels a change of 0.56 $\mu\text{amp./}\mu\text{g.}$ was obtained, but at lower levels the effect of fluoride disappeared. This was found to be due to the decrease in the height of the second wave of nitrobenzenearsonic acid, relative to the first, on dilution; the second wave almost disappeared at 10^{-4}M and was about half the height of the first at $5 \times 10^{-4}\text{M}$. This fact, and other evidence detailed in Appendix D led to the

conclusion that the second wave was due to adsorption of the nitrobenzenearsonic acid on the surface of the mercury.

Discussion

There are four factors which must be considered in assessing an analytical method; these are accuracy, sensitivity, specificity and rapidity. To a very large extent, improvement in any one of these factors is likely to be offset by a disadvantageous change in one or more of the other factors. Any useful method is thus a compromise, chosen on the basis of the relative importance of the factors.

In the present work the primary emphasis was placed on sensitivity, with the aim of measuring 0.1 μg . fluoride. However, accuracy was also important, and all operations were carried out to less than 1% error in the anticipation that this would lead to a final error of little over 1%. Clearly the final error is a function of the number of operations to be carried out (i.e. the rapidity) and

shortening a method should, from this point of view, increase the accuracy, although other effects may swamp the change. Furthermore, a short method is less liable to accidental errors and can be more easily rechecked if necessary. Nevertheless a lengthy method would be acceptable if it could provide a sufficient level of accuracy.

In the first method examined, the major portion of the time required to complete a run was occupied by incubation of the samples, which required no attention during this interval. The actual working time was not long, and the procedure was found to be suitable for the analysis envisaged, but it was less satisfactory for experiments designed to improve the accuracy, where one prefers to design each experiment on the basis of the results of the previous one.

Several other workers have used diffusion bottles for separating fluoride: in most cases larger amounts of fluoride were used, but all claimed diffusion was complete in twenty-four hours or less. Stegemann¹⁷² measured 4 - 8 μg . fluoride after sixteen hours diffusion, but did not give any measure of variability. Buttner³³

also used sixteen hours and obtained a standard error of 0.2 $\mu\text{g.}$ with 5 - 30 $\mu\text{g.}$ fluoride added. The method has been refined considerably by Hall⁷⁹, who obtained 96% recovery of 0.1 $\mu\text{g.}$ with a standard deviation (calculated from the results given) of $\pm 0.0083 \mu\text{g.}$, and better values at somewhat higher levels.

It is significant that the originators¹⁶² of the diffusion method have not used it extensively in their later publications, and have recently suggested¹⁶⁴ that a polypropylene Conway unit is more suitable, although no figures are given. The use of a Conway unit has been recorded in a number of recent investigations. Greenland⁷⁵ obtained an error of 4.6% with 2 - 10 $\mu\text{g.}$ fluoride. Frere⁶³ claimed 93 - 95% recovery, with a precision of 2%, diffusing 1 - 50 $\mu\text{g.}$ fluoride at room temperature. However Wharton¹⁸⁵ disputed this and stated that incubation for 24 hours at 60°C. was necessary for 0.5 - 3 $\mu\text{g.}$; the standard deviation under these conditions was $\pm 0.03 \mu\text{g.}$ It appears that the Conway cell is likely to prove more suitable for small amounts of fluoride, but there is still scope for refining the technique.

The second method described is entirely novel, both as regards the reagent employed and the mechanism of the reaction; arsonic acids are standard reagents for the gravimetric analysis of thorium, but no record of their use in polarographic analysis has been found in the literature. The method provided a convenient and accurate determination of microgram amounts of fluoride, but is unfortunately not sufficiently sensitive for the present purposes.

As for specificity, all fluoride methods show interference from substances which complex fluoride, principally Al, Fe, Sn, Zr and Th. These are present in dental tissues at low concentration and are unlikely to be the source of any difficulty. All fluoride methods based on metal chelation are in addition susceptible to interference from substances which form complexes with the metal used. The two principal materials in this class which occur in dental tissues in relatively large amounts are phosphate and citrate. For this reason any method using a metal in the final solution requires either diffusion or distillation before the estimation. The accuracy of measurement should, however, be

increased by eliminating this stage; consequently methods of fluoride measurement not requiring any metal should under favourable conditions not require diffusion. Two such methods of requisite sensitivity are known: the adsorption-isotope dilution method⁷⁶, has the overriding disadvantage of necessitating a cyclotron in the basement, owing to the short half-life of ^{18}F ; the other method⁷⁷ is enzymic and is described in the next chapter.

CHAPTER 4

ENZYME STUDIES

Enzymes are now used in a number of standard methods of analysis. The general principle is to select an enzyme which uses as a substrate the substance which is to be determined, and to couple this reaction either directly or indirectly with a reaction involving another substance which can be readily measured, usually a spectrophotometrically. The initial reaction proceeds to completion and the product should be formed stoichiometrically. The conditions are thus favourable for obtaining a high accuracy, and with a suitable choice of enzymes, a high specificity may also be obtained.

The only enzyme at present known to use fluoride as a substrate is pyruvic kinase¹⁷⁹ (2.7.1.40)¹ which has been shown to catalyse the reaction:



An assay method has been described¹⁷⁹ for the enzyme in which phosphoenolpyruvate, lactic dehydrogenase and NADH are added, and the

disappearance of NADH is followed spectrophotometrically. Pyruvic kinase is now available commercially and by adding it to the system, fluoride activation could readily be coupled to NADH oxidation.

The molar extinction coefficient for NADH at 340 mμ is $6,220^{87}$, so that a change in optical density of 0.1 is equivalent to a change in concentration of $16.1 \mu\text{M}$. In an ordinary 1 cm., 4 ml. cell this corresponds to $0.064 \mu\text{moles}$ or $1.2 \mu\text{g. fluoride}$. Thus as a method for fluoride analysis, the sensitivity is not adequate for the present work. The sensitivity could be increased by using micro-cells, although there is then a loss in accuracy due to the difficulty of measuring small volumes; the sensitivity might also be improved by the use of fluorimetry⁷⁴.

The method has many advantages where a high sensitivity is not required and it is hoped to examine it in the future. The principal problem to be anticipated would appear to be contamination with pyruvic acid of bacterial origin.

Another method of using enzymes for analysis involves the measurement of changes in

the rate of the reaction produced by the substance to be measured. Such kinetic effects have been utilised for analysis in inorganic systems¹⁵³~~152~~, but have never been employed widely probably because of the liability of reactions to inhibition by a variety of materials. This is certainly the primary disadvantage in using enzymes but it can be overcome in two ways: either the components of the system must all be known both qualitatively and quantitatively and their effects on the enzyme determined, or the composition of the system must remain constant through each set of determinations (apart from the substance being measured). In the present investigation the major components were known, but an endeavour was made to maintain the composition constant in order to prevent any interference from minor components.

The advantage of this method is that one is using a chemical amplifier which provides a much higher sensitivity: each molecule of inhibitor, reacting with one molecule of enzyme is able to influence the rate of formation of large numbers of molecules of the product. This may be offset at high dilutions by dissociation

of the enzyme-inhibitor complex and the limit of usefulness is in practice set by the kinetic characteristics of the enzyme.

In the literature, two enzymes have been suggested for fluoride analysis by virtue of their high sensitivity to fluoride inhibition: liver 'lipase' ⁹~~14~~ and potato acid phosphatase ¹⁷³~~172~~ (3.1.3.2.). No confirmation of these reports was available at the time this investigation was started, apart from a very brief note ¹⁵⁵~~154~~, but it was necessary to decide which enzyme to use since a considerable amount of preliminary work was necessary. Liver 'lipase' had two advantages: it was claimed that it was unaffected by phosphate ⁵⁰~~51~~, and the sensitivity was reported as being the higher of the two; furthermore it was felt that knowledge of the biochemistry of this type of enzyme activity was at a potentially more interesting stage. The value of liver 'lipase' for fluoride analysis has since then been confirmed ¹⁰⁷~~106~~.

It was claimed ¹¹¹~~110~~ that liver 'lipase' could be inhibited 50% by sodium fluoride at a dilution of 1 in 5,000,000 i.e. 0.1 p.p.m. F. In addition to the analytical possibilities of

this statement, it is of interest from two other points of view. (1) Lipases and esterases are considered to be non-metallic enzymes¹³⁴ and no organic structure is known with a high affinity for fluoride ion. The characterisation of the active site of the enzyme might therefore lead to a new reagent for fluoride. (2) The concentration of fluoride in blood is stated to be in the region of 0.1 p.p.m.¹⁶³ and analyses of soft tissues⁸¹ indicate a similar concentration in cell water. It is thus of interest to enquire what function an enzyme can perform in the presence of a substance able to inhibit it almost completely. Possible explanations cannot be discussed profitably at the present stage, but it may be relevant that Hall has claimed⁷⁹ that the fluoride content of blood is much lower than at present indicated by authorities.

Substrate

Having selected the enzyme it was next necessary to select a substrate. The substrate

of liver 'lipase' was ethyl butyrate (the enzyme is now classified as an esterase (3.1.1.1.)); the estimation depends on the titration of liberated hydrogen ion and cannot be used in the presence of buffers. Indoxyl acetate¹⁸⁰ was tried, but the indigo produced was adsorbed strongly onto the protein present and no solvent could be found to extract it completely. At a later stage indoxyl acetate was again tried with a clear enzyme solution, but was finally abandoned because it showed an induction period; ferricyanide, which might have abolished this, was inhibitory.

A further point also became evident: it was necessary to dissolve indoxyl acetate in an organic solvent in order to test it, and to use the enzyme in an untreated cell homogenate. Thus it was possible that there might be sufficient solvent to inhibit the most useful enzyme, although other esterases might not be inhibited. It was clearly desirable therefore to use a soluble substrate. Carboxylic substrates (e.g. aspirin⁸⁵) would probably have restricted the pH range by insolubility in acid, and a substrate containing a sulphonate

group appeared preferable.

Two other main types of substrate for esterase have been suggested, both of which can be sulphonated. The indophenol dyes used as redox indicators (and usually sulphonated already) can be acetylated and form useful substrates⁹⁶. However, these dyes are also pH indicators, which would complicate the estimation over a wide pH range. Various substituted naphthyl acetates are used extensively in histochemical methods for esterases, by coupling with diazonium salts. In this application, low solubility is sought, so that soluble substrates are not available commercially. The extensive investigation by Gomori^{70,71} was found useful in selecting the most suitable type of compound, and a simple β -naphthol carrying a sulphonate group on the opposite side of the ring was selected.

The compound sodium 2-naphthol-6-sulphonate was acetylated by heating 20 g. (0.08 mole) on a water bath for 4 hours under reflux with 5 g. (0.06 mole) anhydrous sodium acetate and 60 ml. (0.6 mole) acetic anhydride. The mixture was poured into

ethanol to precipitate the ester, and the resulting solid was extracted with hot 70% ethanol to remove sodium acetate.

Recrystallisation presented some difficulty since any solvent containing water caused hydrolysis and the product was not soluble in non-aqueous solvents. The product was eventually recrystallised from glacial acetic acid containing a few drops of water, with only slight heating to dissolve it initially, until the colour produced on adding the diazonium salt was reduced to a constant minimum.

The final product showed only a slight reaction with diazonium salt; after incubation with enzyme solution a much greater reaction was obtained. Taken together with the unambiguous method of synthesis, this evidence was considered sufficient to characterise the substance as 2-acetoxynaphthalene-6-sulphonate. Some evidence as to purity was obtained from an elementary analysis kindly carried out by Dr. J.W. Minnis: C, 50.17%; H, 3.32%; Na, 7.5% (from residue assumed Na_2SO_4); S, 9.18%; O (difference), 29.83%; required for $\text{C}_{12}\text{H}_9\text{SO}_3\text{Na}$; C, 50%; H, 3.1%; S, 11.1%; Na, 8%; O, 27.8%.

The data suggest that the original material was deficient in sulphur, which was replaced by oxygen without reducing the acid groups. It is difficult to suggest how this could be done, and further confirmation might be useful. It is clear, however, that no water of crystallisation is present, and, assuming that all molecules are acetylated, the molar concentration can be calculated with little error. In fact, the size of the blank value produced in the enzyme tests by solutions of the substrate indicates that about 2% unacetylated material is present.

Measurement of enzyme activity

The enzyme reaction was carried out in phosphate buffer, adding tris or acetate as necessary for wider pH ranges. It was then necessary to stop the reaction and adjust the pH to a constant value for all tubes before adding the diazonium salt, as the rate of coupling varies with pH. Various methods of stopping enzyme reactions in neutral solution were considered

and zinc precipitation to adsorb the protein appeared to be the simplest. The solution was not entirely clear however, and after coupling acid was added. (Heating could not be used for inactivating the enzyme as there was appreciable non-enzymic hydrolysis of the substrate).

Method I: To each sample, made up to 1.8 ml., was added 0.5 ml. buffer containing 0.4 M acetate, 0.4 M phosphate and 1.2 M potassium (adjusted to the required pH with hydrochloric acid) and 0.4 ml. of a suitable dilution (usually fiftyfold) of homogenate. The enzyme action was started by adding 0.2 ml. 0.1% substrate and the tube was incubated at room temperature for 15 min. The enzyme action was stopped with 0.6 ml. solution containing 0.05 M zinc ion (added as zinc oxide) and M maleic acid adjusted to pH 6.5 with potassium hydroxide, producing a precipitate of zinc phosphate. 0.5 ml. 0.12% diazonium salt of p-nitraniline (dissolved initially in acetone to a final concentration of 10%) was added, colour development being complete in less than 5 min. Finally 0.5 ml. 70% trichloroacetic

Table II over

T A B L E II

Extinctions at specified wavelengths in
0.1 M sodium dihydrogen phosphate

Wavelength mμ	2-acetoxy- naphthalene- 6-sulphonate 0.0266%	2-naphthol- 6-sulphonate 0.0224%
400	0.010	0.007
350	0.012	0.017
340	0.018	0.220
334	0.035	0.887
332	0.041	1.107
330	0.046	1.153
326	0.066	0.928
320	0.276	1.089
310	0.355	1.205
	0.00266%	0.00224%
300	0.072	0.271
280	0.472	0.543
260	0.446	0.299
250	0.300	0.256
244	0.298	1.246
242	0.383	2.04
240	0.611	2.53
236	1.89	

[To face page 78

acid was added, the tube was centrifuged and the optical density was measured at 487 mμ.

This method was useful in the presence of large amounts of contaminating protein, but was not sufficiently accurate for kinetic work. The reason for this was not examined, but it was found later that the substrate was unstable in acid. The possibility of using the ultraviolet absorption of the substrate (as suggested by Hofstee for aspirin⁸⁵) was therefore investigated; the measurements are shown in Table II.

It was concluded that the free naphthol could be conveniently and accurately measured in the presence of its acetate from the absorption maximum at 331 mμ. Furthermore an increase of sensitivity of about twentyfold, with some loss of accuracy could be obtained from measurements at 242 mμ, although this latter has not so far proved necessary.

The absorption shown by the substrate at 331 mμ includes the absorption of the impurity present and the shape of the absorption curve suggests that about 2% impurity still remains. Further work might result in a slight improvement.

The substrate showed a definite non-enzymic

hydrolysis at pH greater than 7.5. This property was utilised to check the extinction after complete hydrolysis. 26.5 mg. substrate was incubated in 25 ml. 0.01 N sodium hydroxide. 0.6 ml. was removed at intervals, 0.75 ml. 0.4 M phosphate buffer pH 2 was added and the solution made up to 4 ml. The results were:

Time (min.)	Extinction at 331 m μ
20	0.652
30	0.651
40	0.670
60	0.674

After 18.5 hr. 5 ml. of solution and 9.375 ml. phosphate buffer were made up to 50 ml. The extinction at 331 m μ was 0.459. This is equivalent to an extinction of 0.433 for a concentration of 0.01%. The reaction was thus 98% complete after one hour. The result of this experiment has been used whenever it has been necessary to calculate the absolute amount of substrate or product from the extinction.

The existence of a characteristic spectral absorption of the product of an enzyme reaction is a very convenient means of following the

Table III over

TABLE III

Effect of acid on enzyme reaction

Each tube contained:

0.6 ml. 0.1% substrate

0.75 ml. enzyme (Preparation 10/3B diluted
50x) (where specified)

Acid as specified

Water to make 3 ml.

Incubated 2 hr. except blank which was measured
immediately after mixing

meq. HCl added	Extinction at 331 mμ	
	No enzyme	With enzyme
0.1 (blank)	0.025	0.046
0.01	0.026	0.050
0.03	0.025	0.046
0.1	0.032	0.052
0.3	0.042	0.067
1.0	0.082	0.111

reaction, and can be used in two ways: either continuously, in the spectrophotometer cell, or after stopping the reaction at a particular time. The time course of the enzyme reaction was examined, but was not used for most of the work owing to the long incubation required. Furthermore, in work with fluoride it was desirable to avoid carrying out the reaction in silicon-containing vessels.

For stopping the reaction trichloroacetic acid was first used, but with purified enzyme solutions (3.1.1.2 and 6), there was so little precipitate that it failed to pack adequately in the centrifuge, and hydrochloric acid was preferred for clear solutions, as no precipitate was produced. The blank, however, appeared to increase with time so the effect of various concentrations of acid was investigated. (See Table III). It was concluded that with 0.03 meq. of acid there was no appreciable non-enzymic hydrolysis nor any enzyme action. This solution was found to have pH 2, which was a fortunate coincidence since the first dissociation of phosphoric acid has a $pK = 2$ approximately; the precise adjustment is

therefore not very critical and small variations in the buffering capacity of the reagents are not of great importance. The final procedure adopted for measuring the enzyme activity was as follows:

Method II: In a polythene tube were placed in order 0.75 ml. 0.4 M phosphate buffer (pH 6.5 unless otherwise specified), water to give a final volume of 3 ml., 0.75 ml. enzyme solution suitably diluted and the sample containing fluoride. The tubes were placed in a water bath at 25°C. and 0.6 ml. 0.1% substrate was added to start the reaction, the contents being mixed by lateral shaking. After 1 hr., 1 ml. 0.34 N hydrochloric acid was added and the extinction was measured at 331 mμ.

Enzyme preparation

The original description¹¹¹ for the preparation of the enzyme was taken as a model, but grinding with sand was replaced by homogenisation. 1/2 lb. of pig liver was cut up, placed in a Waring blender with 800 ml. water

and homogenised for 1 min.

Having established the conditions for measurement, the initial investigation was concerned with the effect on pH:

Preparation 3/0 (whole homogenate), final dilution 250x

Method I, 0.2 M tris/acetate buffer, incubation 15 min. at room temperature.

Activities in arbitrary units corrected for blank

pH	4.5	5	5.5	6	6.5
Activity	0.19	0.20	0.22	0.25	0.27
Activity with 1 μ g.F/ml.	0.07	0.115	0.15	0.16	0.23

In this preparation the activity rises progressively with pH (in a somewhat similar manner to the activity of crystalline pig liver esterase⁵) and the effect of fluoride becomes greater at lower pH.

Owing to this effect and to the non-enzymic hydrolysis of substrate in alkaline solutions and the desire to eliminate any effects of phosphate, a new buffer was prepared and compared with the

previous one:

Preparation 4/0 (whole homogenate) final
dilution 500x

Method I, incubation 15 min. at room temp.

Activities in units of optical density
corrected for blank

Buffer pH	0.2 M tris/ acetate	0.2 M phosphate acetate
4.5	0.08	0.11
5.0	0.12	0.18
5.5	0.17	0.47
6.0	0.22	0.29
6.5	0.40	0.39
7.0	0.49	0.37
7.5	0.50	0.52
8.0	0.58	0.58
8.5	0.50	0.47

It appears that an enzyme is present with a pH optimum at pH 5.5, which is inhibited by trishydroxymethylaminomethane. The marked peak appeared consistently in this particular preparation but has not been observed in others (as in the previous table). This is clear evidence that the esterase activity of liver is heterogeneous: this has also been demonstrated by electrophoresis¹⁴³~~144~~, but has not been generally appreciated.

Purification was therefore clearly desirable and for this, a method of measuring the amount of contaminating protein was required, rapidity being

Table IV over

TABLE IV

Measurement of organic content of homogenate

1 ml. diluted homogenate

3 ml. 17 mM sodium dichromate in
concentrated sulphuric acid

Heated in boiling water for 20 min.

Dilution	Optical densities			R x dilution
	440 mμ	650 mμ	650 mμ less blank (R)	
∞	1.10	0.135	0	
100	1.11	0.285	0.150	15.0
50	1.10	0.427	0.292	14.6
25	0.67	0.600	0.465	11.6
10	1.05	0.79	0.66	6.6
5	3.0	1.8	1.7	8.5

R is the relative organic content

in this case more important than accuracy. For this reason the procedure of Johnson⁹⁴~~100~~ was examined (see Table IV). The original method specified absorption measurements at 440 mμ i.e. the disappearance of the dichromate colour was measured. However, no change could be detected at this wavelength with small amounts of homogenate, but the appearance of the green colour of the chromic ion could be measured readily in the same solutions at 650 mμ. The calibration becomes more curved as the amount of material increases, but providing measurements are restricted to the middle of the range, a rough assessment of the organic content is available. The original method⁹⁴~~100~~ has not been used much⁴⁷~~48~~: the results quoted may be the reason for this.

Purification of enzyme

Following preliminary experiments the first stage was an examination of the high-speed supernatant, which showed only weak fluoride

inhibition:

Preparation 9/3W (supernatant after
centrifuging for 30 min. at 90,000 g)
final dilution 125x

Method I, buffer pH 5.5, incubated 15 min. at
room temperature

Activities in optical density at 487 mμ
corrected for blank

μg. F/ml.	0	0.05	0.1	0.19	0.38	0.63
Activity	0.155	0.17	0.17	0.145	0.16	0.15

μg. F/ml.	0	1	2	5	10	25
Activity	0.13	0.105	0.11	0.105	0.105	0.07

Inhibition was only clearly apparent at
25 p.p.m. F, although it is possible that a minor
component of the activity is inhibited down to
1 p.p.m. F. Attention was therefore turned to
the particulate fraction and the activity was

[P.T.O.]

compared with the original homogenate:

Preparation 9, final dilution 50x for organic
content 250x for activity

Method I for activity, pH 5.5, incubated
10 min. at room temperature

All units in optical density corrected for
blank

Fraction	Activity	Organic content	Specific activity
Original homogenate	0.14	0.27	0.52
Supernatant after 1000 g 30 min.	0.18	0.27	0.67
Sediment after 90,000 g 30 min.	0.065	0.11	0.59

Since the specific activity has not altered significantly, there would appear to be prima facie, no purification by this procedure. It has however been shown that the supernatant enzyme is less susceptible to fluoride than the homogenate, so that the procedure must separate two enzyme activities of similar specific activity.

In order to obtain a preparation more stable to storage, the effect of freeze-drying on the particulate fraction was investigated. The activity on reconstitution the following day was similar to that of the original fraction, but

over a period the activity gradually dropped, and this procedure was not pursued. The effect of fluoride was however determined on the frozen-dried preparation:

Preparation 8/2FD (frozen-dried particulate fraction, reconstituted) final dilution 125x

Method I, incubation 15 min. at room temperature

Activities in optical densities corrected for blank

Buffer pH	5	5.5	6
No fluoride	0.04	0.06	0.09
With 0.25 p.p.m. F	0.015	0.025	0.045

Although the activity is rather weak, the figures show that an effective inhibition is present with a low level of fluoride.

Further progress with purification was unlikely to be very great with an insoluble preparation, and methods of solubilising the enzyme were considered. It has been claimed that esterase can be solubilised with alkali⁵⁵, but it was not clear which enzyme was involved. A more general method is butanol extraction¹³², and this was found to be very satisfactory:

The sediment after centrifuging at 90,000 g for

10 sec. in 1/3 of the original volume of cooled water and n-butanol was added with stirring over a period of 30 min. at 4°C. to make a total concentration of 20%. After stirring for a further 30 min., the mixture was centrifuged at 90,000 g for 30 min. The layers of butanol and precipitated protein were removed and the aqueous layer was extracted gently with toluene. The solution was stored in a refrigerator under toluene.

The effectiveness of the whole procedure was examined by measuring the activity and organic content of each fraction. Some difficulty was experienced in completing the estimations before bacterial attack on the homogenates had started, in spite of storage under refrigeration, and only one complete set of data is available:

[P.T.O.]

Preparation 10

Method I for activity, incubated 15 min. at room temperature

All figures in arbitrary units

Fraction		H	L	S	E
Final dilution		125x	125x	42x	42x
Organic content		0.60	0.57	0.9	0.12
Relative activity	(pH 5.0	0.073	0.152	0.079	0.265
	(pH 5.5	0.071	0.252	0.126	0.375
	(pH 6.0	0.174	0.260	0.198	0.355
Specific activity	pH 6.0	0.29	0.46	0.22	3.0

H = Whole homogenate
L = Low speed supernatant
S = High speed sediment
E = Butanol extract

The specific activity of the high speed sediment, as has been shown already, is roughly similar to that of the original homogenate, but the activity in this sediment represents less than a quarter of the whole, so that it cannot be expected to have a very great effect on the properties of the whole homogenate. The organic content of the butanol extract was measured after drying on a water bath to eliminate the toluene

used as preservative, and is considerably higher than in other preparations. In one case the organic content of the butanol extract was reduced to 1.5% of the original, but it was not possible to compare the activities. There is also a very marked activation by butanol extraction, presumably due to removal of access limitation by the material to which the enzyme was originally bound; however, it will be shown that organic solvents have an activating effect. In the experiment above, the activity at pH 5 was increased from 0.265 to 0.385 by the inclusion of 2% acetone in the incubation mixture. This effect together with the multiple nature of the esterases present makes the specific activity a somewhat unreliable guide to the degree of purification achieved.

Properties of enzyme extracts

In the first place the level of fluoride inhibition was examined:

Preparation 9/2WB (butanol extract) final dilution 62x

Method I, pH 5.5 incubated 15 min. at room temperature

Activities in optical densities at 487 mμ corrected for blank

Final F μg./ml.	0	0.05	0.1	0.2	0.3	0.4
Activity	0.275	0.175	0.105	0.09	0.075	0.065
Inhibition %		36	62	68	73	76

Under these conditions the level for 50% inhibition appears to be in the region of 0.07 p.p.m. F, which provides effective confirmation of the original report^{III}. As already indicated, however, the results obtained with this method are not sufficiently accurate for quantitative analysis.

It was also found that 1 mM EDTA had no effect during incubation or when pre-incubated with the enzyme. The effect of glutathione was

more interesting:

Preparation 9/2WB (butanol extract) final
dilution 62x

Method I, pH 5.5, incubated 15 min. at room
temperature

Activities in optical densities at 487 mμ
corrected for blank

	Activity
No addition	0.191
0.6 μM/ml. glutathione during incubation	0.085
Enzyme pre-incubated 3 hr. with 1 μM/ml. glutathione	0.197

Glutathione is thus inhibitory during incubation but not if pre-incubated with enzyme. While numerous explanations are possible, the simplest is that the esterase is also a peptidase and the inhibition is competitive. During pre-incubation the glutathione is almost certainly hydrolysed, since peptidases may be expected to be present apart from the esterase. Many proteolytic enzymes are known to have esterase activity: the esterase action of chymotrypsin has been investigated extensively²².

Table V over

Fig. 2

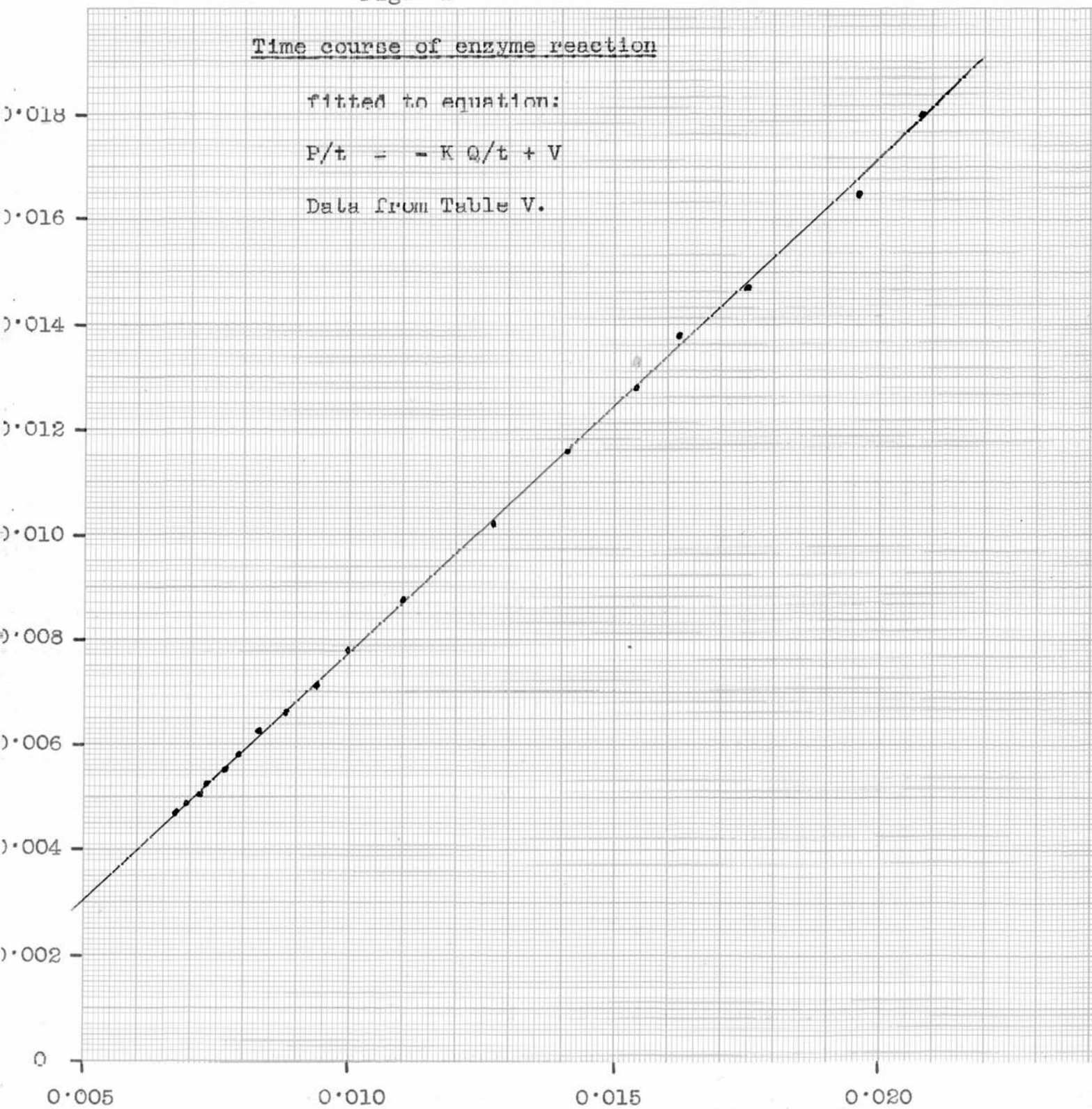


TABLE V

Time-course of reaction

0.6 ml. enzyme (Preparation 10/3B) final
dilution 42x

0.75 ml. 0.4 M phosphate/acetate buffer pH 5.5

1.35 ml. water

Reaction started with 0.3 ml. 0.2% substrate

t	P	P/t	Q/t
1	0.018	0.018	0.0208
2	0.033	0.0165	0.0196
3	0.044	0.0147	0.0175
4	0.055	0.0138	0.0162
5	0.064	0.0128	0.0154
7	0.081	0.0116	0.0141
10	0.102	0.0102	0.0127
15	0.131	0.00873	0.0110
20	0.156	0.00780	0.00995
25	0.178	0.00712	0.00936
30	0.198	0.00660	0.00880
35	0.215	0.00624	0.00824
40	0.232	0.00581	0.00783
45	0.248	0.00552	0.00765
50	0.263	0.00526	0.00730
55	0.279	0.00507	0.00718
60	0.292	0.00487	0.00692
65	0.306	0.00471	0.00669

t = time in min.

P = extinction of solution at 331 mμ
corrected for blank

Q = $\ln S_0/S$

S₀ = initial substrate concentration

S = substrate concentration after time t
(i.e. S₀ - P)

[To face page 93]

Kinetics

The kinetics of the reaction were next investigated using the ultraviolet absorption of the substrate. The extinction was recorded at intervals and an example of the results is shown in Table V and Fig. 2. The figures were analysed by means of the integrated Michaelis equation:

$$\frac{P}{t} = -K \frac{Q}{t} + V$$

K and V being constants; the other terms are defined in Table V.

It is clear that the points lie on a straight line within the experimental error, and that for practical purposes the equation is obeyed. During the course of the run the substrate concentration was reduced by a factor of 0.65. If two different enzymes were present the graph should show curvature; it is therefore concluded that if a second enzyme is present, either its Michaelis constant coincides with that of the first enzyme, or that the constant lies well outside the range of substrate concentration examined. In either case the preparation is

Table VI over

T A B L E VI

Calculated values for K and V at different
substrate concentrations for equation:

$$\frac{P}{t} = -K \frac{Q}{t} + V$$

Initial substrate concentration (S ₀) %	0.01	0.02	0.05
Extinction at equilibrium (calculated)	0.433	0.866	2.165
K	-0.471	-0.930	-2.91
Standard error of K	0.0032	0.0018	0.043
V	-0.00131	-0.00154	-0.00434
$\frac{1}{V}$	-763	-649	-230
$\frac{K}{V}$	360	604	671

[To face page 94

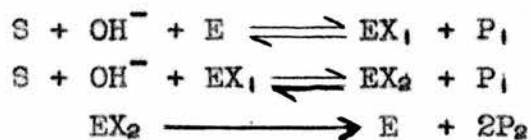
effectively a single enzyme in this range as far as the kinetics are concerned.

The remarkable feature of the graph in Fig. 2 is that both the constant terms are negative. Two other runs were carried out at different substrate concentrations and K and V were calculated in each case by regression of $\frac{Q}{t}$ on $\frac{P}{t}$: the results are shown in Table VI.

It is obvious that one or more of the assumptions on which the Michaelis equation is based do not apply in this case; on the other hand, the fact that the data fit the equation, shown by the low values for the standard errors, suggests that a relatively minor modification should provide an explanation for the negative values of the constants.

A similar situation has been recorded for the action of pancreatic lipase¹⁵⁵~~152~~. The fit of the data to several rate laws was investigated, and the best was the integrated Michaelis equation, which fitted well up to 40% product formation, but thereafter showed a slight but distinct curvature. The curvature was explained on the basis of a three stage reaction, which

represents a simple form of substrate activation:



This leads to a rate equation containing the two terms of the integrated Michaelis equation plus a second order term which is reciprocal function of the substrate concentration, and consequently there are three constants in the equation. The constant corresponding to V (as defined above) was then independent of the initial substrate concentration (S_0) and $\frac{K}{V}$ was proportional to S_0 . The introduction of a third constant not only makes the computation more difficult, but also makes it much easier to fit the data to an incorrect equation.

On the principle of Occam's razor, therefore, an attempt has been made to fit the present results to an equation containing only two terms. A comparison of Fig. 2 with the usual form of the graph ¹⁰¹ shows that the negative constants are due to $\frac{Q}{t}$ decreasing instead of increasing. The rate of increase of Q is therefore slower than expected, which means that there is more

substrate remaining than expected. The reaction is clearly inhibited, but it is important to determine whether the inhibition changes during the reaction, as appears likely at first sight. The general equation for enzyme inhibition is⁶⁴:

$$\frac{V_m}{v} = \frac{K_m}{S} + \frac{K_m I}{K_1 S} + \frac{\alpha I}{K_1} + 1$$

where $v = -\frac{dS}{dt}$
 K_m = Michaelis constant
 V_m = maximum value of v
 K_1 = inhibitor dissociation constant
 I = inhibitor concentration
 α = constant depending on type of inhibition

This equation has been integrated for each of the three cases, putting respectively $I = P$, $I = S$ and $I = \text{constant}$ and the value of the constant terms in the integrated equation are:

	K	V
$I = P$	$\frac{(K_1 + S_0)K_m}{K_1 - K_m + \alpha S_0}$	$\frac{K_1 V_m}{K_1 - K_m + \alpha S_0}$
$I = S$	$\frac{K_1 K_m}{K_1 + K_m}$	$\frac{K_1 V_m}{K_1 + K_m}$
$I = \text{constant}$	$\frac{(K_1 + I)K_m}{K_1 + \alpha I}$	$\frac{K_1 V_m}{K_1 + \alpha I}$

In the first two cases there is an additional term in S^2 which disappears when $\alpha = 0$ (competitive inhibition). It is clear that negative values can only be obtained for K and V when the inhibition increases with the progress of the reaction. This can occur either through product inhibition or through substrate activation. It may be noted that whatever form of substrate activation is assumed, the S_0 term which characterises the coefficients of the integrated equation in product inhibition (and arises from the substitution $P = S_0 - S$) cannot occur in the coefficients of the equation for substrate activation because no such substitution is made. Nevertheless, Table VI showed that K and V are functions of S_0 , so that the effects must be due to product inhibition; substrate activation is not excluded,

TABLE VIIa

Reaction sequences leading to negative constants

<u>Reaction 1</u>	<u>Reaction 2</u>
$E + S \xrightleftharpoons[k_2]{k_1} ES \xrightarrow{k_3} E + P$ $E + P \rightleftharpoons EP \quad K_p = \frac{[EP]}{[E][P]}$ $v = \frac{k_1 k_3 E_0 S}{k_1 S + (k_2 + k_3)(K_p P + 1)}$ $K = \frac{(k_2 + k_3)(1 + K_p S_0)}{k_1 - (k_2 + k_3)K_p}$ $V = \frac{k_1 k_3 E_0}{k_1 - (k_2 + k_3)K_p}$	$E + S \xrightleftharpoons[k_2]{k_1} EP + P_1$ $EP \xrightarrow{k_3} E + P_2$ $v = \frac{k_1 k_3 E_0 S}{k_1 S + k_2 P + k_3}$ $K = \frac{k_3 + k_2 S_0}{k_1 - k_2}$ $V = \frac{k_1 k_3 E_0}{k_1 - k_2}$

Reaction 3

$$E + S \xrightleftharpoons[k_2]{k_1} ES \xrightleftharpoons[k_4]{k_3} E + P$$

$$v = \frac{(k_1 k_3 S - k_2 k_4 P) E_0}{k_1 S + k_4 P + k_2 + k_3}$$

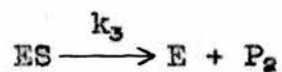
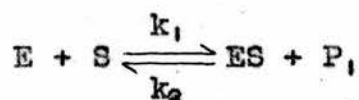
$$K = \frac{k_2 + k_3 + k_4 S_0}{k_1 - k_4} + \frac{k_2 k_4 S_0}{k_1 k_3 + k_2 k_4}$$

$$V = \frac{k_1 k_3 + k_2 k_4}{k_1 - k_4} E_0$$

[Continued in Table VIIb]

TABLE VIIb

Reaction 4



$$v = \frac{k_1 k_3 E_0 S}{k_1 (S + K_p P_1 S + K_2 S^2) + k_2 P_1 + k_3}$$

$$K = \frac{k_3 + k_2 S_0}{k_1 - k_2 + K_p S_0}$$

$$V = \frac{k_1 k_3 E_0}{k_1 - k_2 + K_p S_0}$$

E_0 = total amount of enzyme

E = free enzyme

P = product

v = reaction velocity

K and V defined on page 93

[To face page 98]

and will be shown later to be present.

Product Inhibition

Various types of product inhibition have been listed by Schwimmer¹⁵⁷; only two of these lead to integrated rate equations containing no additional term (in general, additional terms appear to result from postulating more than one enzyme-substrate complex). One further variety was suggested by Kistiakowsky⁵, but not integrated. These schemes are shown in Table VII together with the equations to which they lead. It is felt that the recent recommendations¹ regarding rate constants are unsuitable where the final script is typewritten and a sequential numbering has been used.

The integrated equations have been derived by putting $P = S_0 - S$. In the case of reaction (3), P represents two products and should strictly appear in the rate equation as P^2 : this complicates the integration. Moreover the logarithmic term is slightly different on integrating the rate equation as it stands:

[P.T.O.]

$$- \ln \left[\frac{S}{S_0} + \frac{k_2 k_4}{k_1 k_3} \left(\frac{S}{S_0} - 1 \right) \right]$$

The term $k_2 k_4 / k_1 k_3$ is the equilibrium constant of the reaction, and since the reaction proceeds effectively to completion, the value will be small, and can be neglected during the early part of the reaction. It would seem likely that a term of this kind might account for the deviation recorded for lipase towards the end of the reaction¹⁵⁶.

All the relations for K and V in Table VII are capable of producing a negative value when the rate constant for one of the back reactions is high enough. However, the value of K and V has been shown to become more negative as S_0 increases (Table VI). Reactions (1) - (3) all show this change with K but predict that V remains constant when S_0 changes.

Working back through the integration it was found that in general, the introduction of an S_0 term into the V relation could only occur when an additional S^2 term was also introduced into the equation. One circumstance was found where the S^2 term could be cancelled

out, and this is shown in reaction (4) of Table VII. In the particular case where $K_p = K_2$ the coefficient of S^2 becomes zero. The full equation is:

$$\frac{P}{t} = - \frac{k_3 + k_2 S_0}{k_1 - k_2 + K_p S_0} \ln \frac{S_0}{S} + \frac{k_1 k_3 E_0}{k_1 - k_2 + K_p S_0} - \frac{K_2 - K_p}{2(k_1 - k_2 + K_p S_0)} (S_0^2 - S^2)$$

With this reaction both K and V can become more negative as S_0 increases. It is still predicted (as with the other reactions) that K/V is proportional to S_0 . The results obtained (Table VI) give a curved relation which is suggestive of proportionality between $\frac{K}{V}$ and $\frac{1}{S_0}$: such a relation appears impossible to derive theoretically from reasonable postulates.

The implications of these findings for the mechanism of reaction will be discussed later. From a more practical point of view, the initial velocity fell off so rapidly that it was impossible to obtain more than a rough estimate by the usual method of drawing a tangent. It should be possible using K and V

calculated as described earlier, to obtain the initial velocity v_o from the equation:

$$v_o = \frac{VS_o}{K + S_o}$$

obtained by differentiating the equation $P = Vt - KQ$. Unfortunately, as can be seen from the results of the time-course measurements (Table VI), the value of K is only slightly larger than S_o and opposite in sign. The lower part of the fraction is therefore a small difference and not very accurate, and v_o is correspondingly inaccurate. It was felt at this stage that a more empirical approach would show better the possibilities of the system as an analytical method.

Short Incubation Experiments

The effect of varying the amount of enzyme was examined. The reaction was stopped after ten minutes by adding 1 ml. trichloroacetic acid and the tubes were then centrifuged:

[P.T.O.]

Preparation 10/3B (butanol extract)

Method II, 0.1 M phosphate/acetate buffer
pH 5.5

Final enzyme dilution	42x	100x	167x	250x	500x
Time of incubation min.	5	12	21	30	60
Activity	0.037	0.0295	0.024	0.017	0.014

The time of incubation was adjusted so that if the activity was proportional to enzyme concentration, the same amount of product would be formed in each case. In fact, the activity falls off as the enzyme is diluted. It is probable that this effect is due to the dilution of the organic solvents in the enzyme preparation. It was noted in this experiment that only the highest enzyme concentration showed an appreciable precipitate after adding trichloroacetic acid, and this led to the substitution of hydrochloric acid at this stage.

The effect of varying the substrate concentration was next examined:

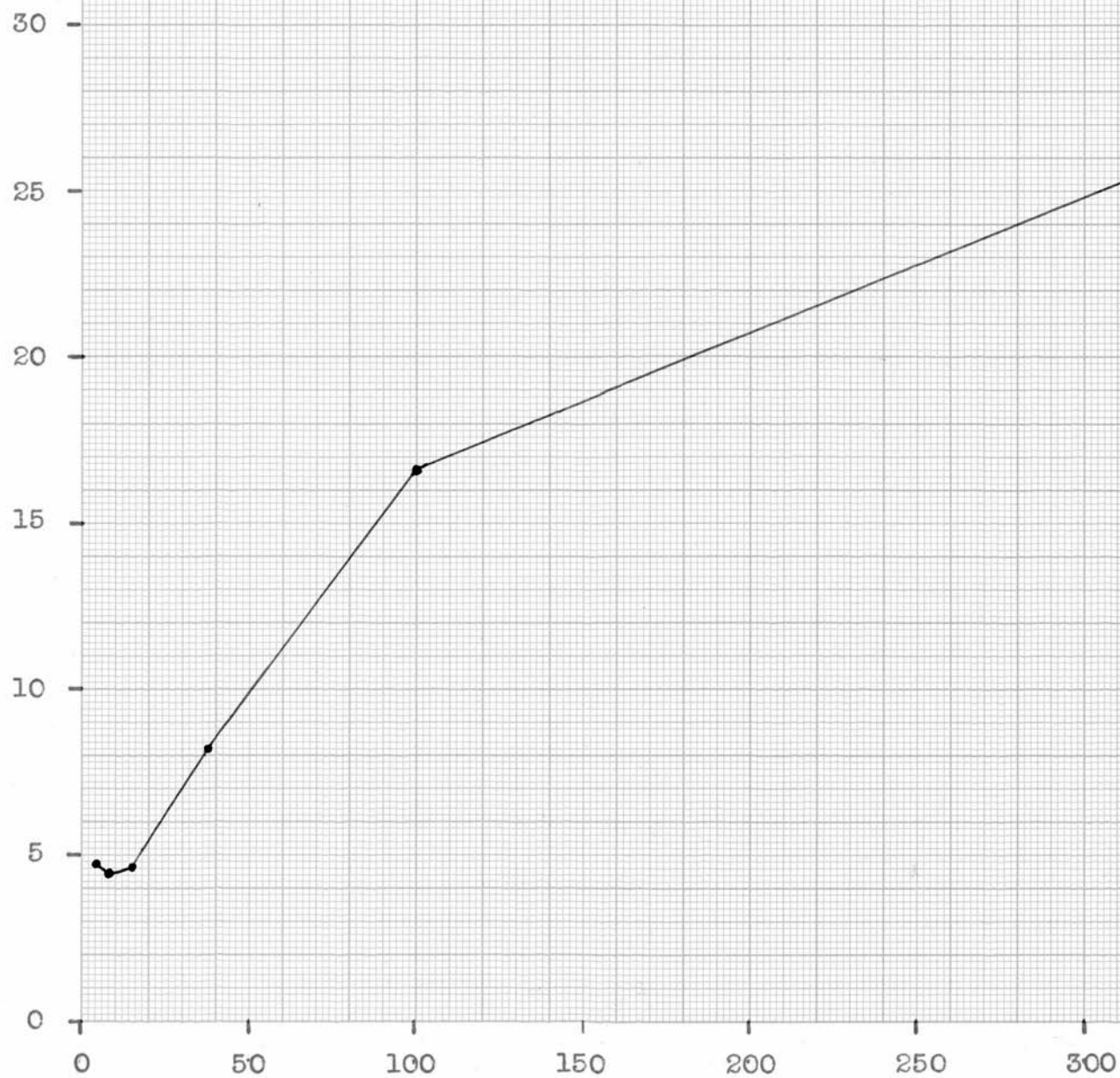
[P.T.O.]

Fig. 3

Enzyme activity at different substrate concentrations

Abscissa: reciprocal of substrate concentration in %

Ordinate: reciprocal of activity



Preparation 10/3B final dilution 62x

Method II, 0.1 M phosphate/acetate buffer
pH 5.5 incubated 7.5 min.

Substrate Final concentration %	Activity
0.24	0.213
0.133	0.225
0.067	0.212
0.027	0.121
0.01	0.060
0.0033	0.039

A Lineweaver-Burk ^{log}~~10~~ plot (Fig. 3) of these results shows a marked curvature that is too large to be accounted for by the correction to initial velocity indicated by the time-course curves.

A graph of this type has been observed for crystalline pig liver esterase⁵, and attributed to substrate activation. It may be observed from Table VII that product inhibition is unable to account for the curvature when the integrated Michaelis equation is obeyed. It is therefore necessary to postulate in addition to product inhibition, some other reaction such as substrate activation in order to explain the results; Kistiakowsky⁵, by a rather different approach has come to a similar conclusion. There is a suggestion of substrate inhibition at high

Table VIII over

TABLE VIII

Effect of pH on enzyme activity

Preparation 10/3B (butanol extract) final
dilution 67x

Method II, 0.1 M phosphate/acetate buffer
incubated 10 min.

Activities in optical densities, corrected
for blank

Final substrate concentration	0.005%	0.04%	0.15%
pH			
3.5	0.011	0.015	0.031
4.0	0.020	0.035	0.045
4.5	0.029	0.071	0.099
5.0	0.027	0.085	0.143
5.5	0.018	0.078	0.158
6.0	0.023	0.098	0.210
6.5	0.042	0.198	0.482
7.0	0.056	0.258	0.690
7.5		0.484	1.179
Equilibrium value (calculated)	0.216	1.732	6.495

[To face page 104

levels in Fig. 3, which is consistent with reaction (4), but further work is required before any definite conclusions can be drawn.

Since the presence of two enzymes in a preparation can lead to a graph of the shape shown in Fig. 3, it seemed better to postpone further analysis of this effect until such time as highly purified preparations were available.

Variation of the pH showed a marked rise in activity above pH 6 (Table VIII). In the interpretation of these results, allowance must be made for the greater reduction in rate in those tubes in which a larger amount of substrate has reacted. The general pattern is similar to that of the original homogenate, but there is a suggestion of a minor proportion of an enzyme with an optimum pH 5.

The effect of pH on the inhibition by fluoride was examined at the lowest substrate concentration since it was anticipated that this would show the highest inhibition:

[P.T.O.]

Preparation 10/3B (butanol extract) final
dilution 67 x

Method II, 0.1 M phosphate/acetate buffer,
incubated 10 min.

Activities in optical densities corrected
for blank

pH	No fluoride	0.1 μ g./ml. F	inhibition %
3.5	0.008	0.009	
4.5	0.023	0.014	39
5.5	0.018	0.008	55
6.5	0.036	0.013	64
7.5	0.070	0.049	30

The fluoride effect appears to have an optimum around pH 6 - 6.5. The decrease in the alkaline range is a common effect with fluoride^{4.8}, but the decrease on the acid side is rather surprising. It seems probable that the enzyme with optimum pH 5 is not inhibited by this level of fluoride, but this point must await further purification of the enzyme for elucidation. It was decided to adopt pH 6.5 for further work, as the best compromise between an effective inhibition and a high activity, and it was therefore unnecessary to include acetate in the buffer. A further experiment showed that decreasing the phosphate concentration in the buffer did not affect the activity, so the same level was continued in use.

An examination of the effect of varying the

Table IX over

T A B L E IX

Effect of fluoride concentration at various substrate concentrations

Preparation 10/3B (butanol extract) final
dilution 62x

Method II, 0.1 M phosphate buffer pH 6.5,
incubated 10 min.

Activities in optical densities corrected
for blank

F	Final substrate concentration					
	0.01%		0.02%		0.04%	
	A	I	A	I	A	I
0	0.043		0.053		0.112	
0.0067	0.037	14%			0.098	13%
0.01	0.0375	19%	0.040	25%	0.091	19%
0.02	0.025	42%	0.035	34%	0.086	23%
0.04	0.019	56%	0.029	45%	0.070	38%
0.05			0.024	55%		
0.06	0.019	56%	0.023	57%	0.060	46%
0.07			0.021	60%		
0.08	0.014	67%	0.022	59%	0.051	54%
0.10	0.012	72%	0.020	62%	0.047	58%
0.12			0.016	70%		
0.15	0.010	77%	0.013	75%	0.037	67%
0.20	0.008	81%			0.034	70%
0.30	0.005	88%			0.026	77%

F = Final fluoride concentration ($\mu\text{g./ml.}$)

A = Activity

I = Inhibition (per cent)

[To face page 106

fluoride concentration showed the general trend to lower inhibition with higher substrate concentration (Table IX). The level for 50% inhibition varies from 0.03 p.p.m. to 0.07 p.p.m. The extinctions were too low for accurate measurement, so a longer period of incubation was tried (Table X).

Two experiments were carried out with an interval of one week between them, the conditions of the second experiment being indentical, within the limits of the procedure, with those of the first. Nevertheless it is immediately apparent that the enzyme activity has almost doubled in the interval and the level for 50% inhibition has increased from 0.06 to 0.17 p.p.m. (approximately). The former value is close to that given by the earlier short period experiment (Table IX). In analysing the results it is of interest to compare the two experiments in Table X in order to specify more closely the change which has occurred, in addition to examining the effect of fluoride.

Since the incubation extended over a period of one hour, initial velocity measurements were not available; moreover the integrated Michaelis

equation has already been shown to fit the time course of the reaction; it was therefore felt that the best fit should be obtained by using an integrated form of the general inhibition equation⁶⁴ (already discussed in connection with product inhibition). Expressed in a suitable form, the integrated equation is:

$$I = \frac{K_1 V t}{K_Q} - \frac{K_1 P}{K_Q} - \frac{\alpha I P}{K_Q} - K_1$$

where I = inhibitor concentration
 $Q = \ln S_0/S$
 K_1 = inhibitor dissociation constant
 α , K and V are constants

The constants in this equation were evaluated by multiple regression of I on P and the values obtained were:

	Experiment	
	1	2
K_1	0.0604	0.0645
K	-0.458	-0.258
α	0.786	0.548
Vt	0.0183	0.1285

It is interesting to note that the value of

Table X over

TABLE X

Kinetics of fluoride inhibition

Preparation 10/3B (butanol extract) final dilution 62x

Method II, incubated 1 hr., 0.02% final substrate concentration

Observed		Calculated from regression equations			
F	P	1	1D	2	2D
<u>Experiment 1</u>					
0	0.266	-0.0081	-0.0081	-0.0157	-0.0157
0.00667	0.209	0.0122	+0.0055	0.0063	-0.0003
0.01	0.204	0.0146	+0.0046	0.0088	-0.0012
0.02	0.181	0.0267	+0.0067	0.0223	+0.0023
0.04	0.165	0.0372	-0.0028	0.0338	-0.0062
0.06	0.129	0.0679	+0.0079	0.0702	+0.0102
0.08	0.115	0.0832	+0.0032	0.0906	+0.0106
0.10	0.111	0.0909	-0.0091	0.0973	-0.0027
0.15	0.083	*	-0.0264	0.1628	+0.0128
0.20	0.069	0.2377	+0.0377	0.2155	+0.0155
0.30	0.058	0.2803	-0.0197	0.2748	-0.0252
Standard deviation		±0.0168		±0.0095	
		(n = 10)		(n = 11)	
<u>Experiment 2</u>					
0	0.425	0.0033	+0.0033	0.0075	+0.0075
0.01	0.415	0.0068	-0.0032	0.0111	+0.0011
0.025	0.372	0.0235	-0.0015	0.0283	+0.0033
0.05	0.311	0.0491	-0.0009	0.0621	+0.0121
0.10	0.285	*	+0.0124	0.0807	-0.0193
0.15	0.227	0.1395	-0.0105	0.1374	-0.0126
0.20	0.185	0.1990	-0.0010	0.2006	+0.0006
0.30	0.141	0.3014	+0.0014	0.3074	+0.0074
Standard deviation		±0.0065		±0.0100	
		(n = 7)		(n = 8)	

F = Final fluoride concentration (μg./ml.)

P = Product formed (arbitrary units)

1 = Values of F calculated from regression using equation:

$$a = b \ln S/S_0 + cP \ln S/S_0 + dFP \ln S/S_0 + F$$

(S = S₀ - P; S₀ = 0.6255 in units of P)

1D = Difference between 1 and F

2 = Values of F calculated from regression using equation:

$$F = m/P + k$$

2D = Difference between 2 and F

n = Effective number of observations

* = Equation becomes indeterminate at this point; deviation estimated from sum of other deviations

[To face page 108]

K_i has changed very little between the two experiments, which gives one some confidence that the value is correct. However, this is offset by the fact that α is fractional, and has changed by a considerably larger amount. A fractional value of α normally indicates a mixed competitive and non-competitive form of inhibition, since α is related to the relative affinity of substrate for the inhibited and uninhibited forms of the enzyme. It thus appears that either the Michaelis constant of the enzyme has changed or the equation is not applicable. Furthermore, although the value of K is negative as found previously, the value of V is positive, and none of the reaction schemes discussed earlier is capable of explaining such values without postulating a third term in the equation.

The fit of the regression line is shown in Table X, column 1D: the deviations are relatively small except in one region close to where the equation becomes indeterminate. This effect occurs in both experiments, and suggests that the equation is not suitable for describing the results.

Many types of inhibition give a straight line with a simple reciprocal plot of the reaction velocity. Therefore a simple linear regression of the data was carried out using the equation:

$$F = \frac{m}{P} + k$$

giving the following values with standard errors:

		Experiment	
		1	2
m	0.0216 ±0.00096	0.0633 ±0.0026	
k	-0.097 ±0.0091	-0.141 ±0.0186	

and the expected fluoride concentrations are listed in Table X (col. 2). Expressions for m and k contain at least three constants, which cannot therefore be evaluated quantitatively. The fit of this equation to the data is shown in column 2D of Table X. The standard deviations are rather high, but the scatter does not show any consistent pattern.

A comparison of the fit of the two equations shows little to choose between them. The variance ratio of the two experiments using the four-parameter equation is 6.6 which is significant at the 1% level. The variance ratio

for the two equations in Experiment 1 is 3.15 which is just significant at the 5% level. None of the other ratios is significant. It appears justifiable to conclude that the four-parameter equation does not give a better fit than the two-parameter equation, and that the fit of the former is unreliable. A priori, one would expect a four-parameter equation to give a better fit than a two-parameter equation, if there is any difference between them; when there is no difference in fit, the variance must arise primarily from the data. Therefore, if the variance were to be reduced, a significant difference might appear although this would not necessarily be of importance as regards the mechanism of inhibition. The two-parameter equation is far simpler to use, and further work has made use of it alone.

Unfortunately the simpler equation yields no information about the type of inhibition unless the substrate concentration is also varied and the Michaelis constant is known, except for one fact. The linearity between fluoride concentration and reciprocal of reaction velocity indicates that only one fluoride ion

interacts with each active centre. When more than one ion interacts with the active centre a linear relation is obtained with the appropriate power of the fluoride concentration. This occurs with acid phosphatase, in which case a linear relation with the square of the fluoride concentration was obtained¹⁴⁸.

Analytical possibilities

A check on the reproducibility of Method II gave a coefficient of variation of the extinction of 2.5% without fluoride and 4.1% with 0.1 p.p.m. fluoride using five tubes for each measurement in a single experiment. The standard deviations were similar, so that the variation was to a considerable extent independent of the amount of product formed. This result was considered rather disappointing and an effort was made to obtain some improvement.

The polythene tubes which had previously been cleaned in concentrated alkali were cleaned in chromic acid, and the measurement repeated. The coefficient of variation without fluoride

was 1.45% and with 0.1 p.p.m. fluoride 2.4%. However, this was not maintained and further possible sources of variation were examined. It was shown that the pipetting error was much smaller than this effect, and that stoppering the tubes, or more efficient mixing had no effect. It was noticed in one experiment that the activity in some new polythene tubes was considerably higher; presumably because the polythene had been attacked by chromic acid and was then able to inactivate the enzyme partly. Unfortunately this higher activity showed less inhibition by fluoride. Evidence has already been presented of the variation of the enzyme activity and concomitantly of the level of inhibition (Table X) which is good evidence for the presence of two enzymes in the preparation. It appears that the more labile enzyme is less affected by fluoride. It was clear that at least part of the variation could be explained by this effect, and the opportunity was taken to change over to polypropylene tubes, which are more resistant than polythene, and can be dried very much more quickly. These were cleaned with Lissapol N and gave more satisfactory results.

Nevertheless the uninhibited activity of the enzyme preparation continued to vary from day to day, and it was felt that this effect might influence the variation between tubes. It also made the selection of the optimum conditions for accuracy rather difficult. The further purification of the enzyme therefore appears to be essential to improvement.

The system was used at this stage for fluoride analysis by adjusting the volumes to simplify making up the solutions:

Method III: The sample containing fluoride was placed in a polypropylene tube, weighed, and made up to 1 ml. with water. 1 ml. solution containing enzyme preparation diluted 27x with 0.15 M phosphate buffer pH 6.5 and 3 mM EDTA was added. The incubation was started with 1 ml. 0.05% substrate in the same solution as the enzyme. The solution was mixed quickly and incubated at 25°C for 1 hour. The reaction was stopped with 1 ml. 0.34 N hydrochloric acid and the extinction was measured at 331 mμ. Six standards were normally run with each series of samples.

With this method it was not possible to

Table XI over

T A B L E X I

Reproducibility of enzymic determination

Method III

Activities (P) in arbitrary units

$\mu\text{g. F}$ added	P	$\frac{1}{P}$	F expected	difference
0	0.431	2.320	-0.0085	-0.0085
0	0.425	2.353	-0.0041	-0.0041
0	0.420	2.381	-0.0003	-0.0003
0.0203	0.406	2.463	0.0106	-0.0097
0.0200	0.401	2.494	0.0148	-0.0052
0.0501	0.365	2.740	0.0476	-0.0025
0.0504	0.357	2.801	0.0557	+0.0053
0.0697	0.335	2.985	0.0803	+0.0106
0.0698	0.336	2.976	0.0791	+0.0093
0.1002	0.312	3.115	0.0977	-0.0025
0.1004	0.316	3.165	0.1043	+0.0039
0.1994	0.253	3.953	0.2095	+0.0101
0.2010	0.256	3.906	0.2032	+0.0022
0.5062	0.163	6.135	0.5008	-0.0054
0.5142	0.161	6.211	0.5109	-0.0033

Standard deviation ± 0.0064
(n = 15)

Slope = 0.133

Mean expected F for no
F added and standard
error = $-0.0043 \pm 0.0023 \mu\text{g.}$

Mean F = 0.1268 $\mu\text{g. F}$

Coefficient of
variation of mean F = 5.05%

Coefficient of
variation of mean P = 1.47%

[To face page 114

obtain replicates, since glass pipettes were not used in order to avoid the possible adsorption of fluoride by the glass. The results of one calibration run are shown in Table XI. The values of P are recorded from the extinction of the final solution. The amount of fluoride required to decrease the extinction by 0.1 is 0.092 $\mu\text{g.}$, which is a useful comparative measure of the sensitivity of the method. The corresponding values from Table X are in the region of 0.05 $\mu\text{g.}$ for both experiments, so the enzyme appears to have deteriorated in the interval. On the other hand the standard deviation in Table XI shows a distinct improvement on the values in Table X. The accuracy of estimation of fluoride falls off on either side of the 50% inhibition level, on the lower side because of small differences, and on the higher side because of the properties of the reciprocal function, this is in addition to any change resulting from the regression function. It is therefore important to be able to adjust the conditions to bring the measurement within a fairly narrow range.

One further point must be considered in

Table XI. There is a suggestion of curvature in the last column of differences between observed and expected values. The difference when no fluoride is added is not quite significant at the 5% level. It is not therefore proved that a curvature is present, but it is quite possible that it exists. An attempt to allow for the curvature would hardly be justified unless it was clearly demonstrable.

The effect of toluene in the sample was investigated using this method:

Method III

Activities in optical densities corrected for blank

	No F	0.167 p.p.m. F	Inhibition
No toluene	0.392	0.115	71%
Toluene-saturated water			
0.4 ml.	0.454	0.192	58%
0.8 ml.	0.462	0.224	52%
Pure toluene added	0.471	0.299	37%

Toluene produces a distinct activation, although the effect is not particularly large. There is also a decrease in the effect of

fluoride when toluene is present, suggesting that toluene has activated primarily the less fluoride-sensitive enzyme. The amount of toluene carried into the tube from the enzyme stock is difficult to assess since a few droplets were always present, but these dissolved during dilution of the enzyme and it seems unlikely that the variation in toluene concentration could affect the enzyme activity. In several experiments however, it was necessary to measure the fluoride in toluene-saturated samples, and the standards had to be made up in the same solution as the samples, and equilibrated with toluene before the measurements.

Discussion

The question arises as to whether the enzyme crystallised by Kistiakowsky^{*} is identical with the enzyme described here. No specific statement can be made since the mode of preparation, substrates, inhibitors and activators investigated are all different. It has already been pointed out that there are a

number of esterases present in liver¹⁴⁵~~145~~ so that the probability of obtaining the same enzyme by different isolation procedures and different estimations is small. The only specific property which may have a bearing on the question is that the enzyme isolated by Kistiakowsky, using ethyl butyrate as substrate was inhibited by added butyrate, whereas the enzyme examined here was not inhibited by acetate.

The observation of marked fluoride inhibition in pig liver 'lipase'¹¹¹~~111~~, made over fifty years ago has been amply confirmed, and in fact evidence has been presented which indicates that inhibition occurs at levels even lower than the 0.1 p.p.m. originally claimed, although this is probably due to the use of lower substrate concentrations. An independent confirmation¹⁰⁷~~107~~ has indicated that 0.05 p.p.m. fluoride gave 50% inhibition, but no investigation of the enzyme preparation was recorded.

The enzyme has been shown to be present in an insoluble form in cell homogenates, and a method of carrying out the initial purification and solubilisation has been described. No method of drying the enzyme for preservation has

been found so far, but toluene has been used to preserve the aqueous solutions. This results in some activation of the enzyme.

A new substrate has been prepared for measuring the activity which is water-soluble, in order to avoid the effects of varying concentrations of organic solvents introduced at different substrate concentrations by difficulty soluble substrates. The new substrate has been found to have little absorption of light in the region of one of the peaks of its hydrolysis product, providing a convenient spectrophotometric system for estimation of the enzyme activity. Most workers have used nitrophenyl acetate⁷⁷ for a similar purpose, but phenyl acetate¹⁹ and aspirin⁸⁵ have also been used. The first two are poorly soluble in water, and the last is less soluble in the acid form. Naphthyl esters have also been used, but usually in conjunction with diazotisation and extraction into an organic solvent¹³⁶.

The kinetics of the enzyme activity in the preparation have been examined in some detail, and found to be rather unusual in that the parameters of the equation used turned out to be negative.

This has been shown to be due either to substrate activation or product inhibition. The distinction between these two is not easy when the mechanism is not specified, but in general it appears that the substitution $P = S_0 - S$ in deriving equations for product inhibition leads to a dependence of K on S_0 , but no such substitution is made with substrate activation; however, more complex mechanisms can also lead to K being a function of S_0 , for example when one of the products is not formed irreversibly. If K is found to be a function of S_0 , one can conclude therefore that substrate activation is not an adequate explanation, but it is not thereby excluded. Product inhibition on the other hand explains the dependence of K on S_0 , but not the dependence of V on S_0 and another mechanism must be postulated to explain this.

The experimental data were fitted best by reaction 4 (Table VII). It is not suggested that this represents the actual mechanism of the enzyme reaction, but only that it incorporates in a simplified form the main features of the mechanism: (1) the sequential formation of the products is suggested by the isolation of

acylchymotrypsin and demonstration that it is an intermediate in the hydrolytic reaction¹³. This sequential reaction results in one form of product inhibition (reaction 2) and is required to account for the negative values of K and V ; (2) a second form of product inhibition is required to account for the dependence of K and V on S_0 ; (3) cancellation of the effects of substrate and product, reducing the negative first order (S^2) term to a negligible value. In view of the similarity in structure of substrate and product, it is not unlikely that they should show similar binding to a second site. It is more surprising that two kinds of product inhibition should be required to explain the results; nevertheless the picture of the active site which these reactions suggest is two substrate-binding sites with an acetyl-binding site between: this is what one would expect to be the organisation of an acetyltransferase.

The enzyme activity was later found to vary from one day to another. This suggests some form of inhomogeneity in the preparation, in spite of centrifuging at high speed, and may be related to the presence of toluene causing a

slow precipitation. Further purification is highly desirable and until this has been done, the conclusions above must be regarded as provisional. It appears probable that two enzymes are present, of similar kinetic properties, but differing in their inhibition by fluoride.

The effect of fluoride was found to be described adequately by a simple inverse relation between reaction velocity and fluoride concentration, even after a long reaction time. This was taken as evidence that only one fluoride ion interacted with each active site, but the variation of the apparent Michaelis constant with substrate concentration has prevented any further analysis of the inhibition. Progress in this direction depends on finding a more accurate method for measuring the initial velocity of the reaction.

Since a linear relation was available, lack of knowledge of the mechanism of the inhibition has not hindered the development of an analytical method for fluoride. The greatest difficulty encountered was the variable degree of inhibition of the enzyme, which was apparently

related to the variable activity. This caused difficulty in adjusting the conditions to give the optimum accuracy for the amount of fluoride expected, because the accuracy of measurement, with the reciprocal relation used, falls off on either side of 50% inhibition.

Almost all methods of fluoride estimation, being based on a decrement effect, show a considerably lower coefficient of variation for the actual measurements made compared with that for the amount of fluoride and the methods examined here are no exception: Table X shows that the accuracy is decreased by a factor of 3.44 in converting the readings to amounts of fluoride. Nevertheless, the accuracy attained is not as high as one would expect for an enzyme activity measurement. Some improvement was obtained by standardising the experimental procedure more effectively, but the variation of activity remained rather high. This could not have been due to variations in the amount of fluoride added since this was weighed to 0.5% with the smallest amount. The most likely explanation is that the causes of the variation in enzyme activity found to occur from day to day

also operate to a small extent in a single experiment. It has in fact been shown that part of the enzyme activity is particularly sensitive to inactivation; this labile activity does not seem to be fluoride-sensitive, so that elimination should be possible by further purification, and one would expect this to lead to a decrease in the variability of fluoride measurements.

The primary reason for investigating liver esterase was the prospect of a highly sensitive method of fluoride determination. The most sensitive chemical method available is that of Hall⁷⁹ which has a low blank and a colour development directly proportional to the amount of fluoride. With this method an optical density increase of 0.105 was obtained with 0.4 µg. fluoride; from the figures given the standard deviation of replicates of 0.2 µg. fluoride can be calculated to be ± 0.0045 (coefficient of variation 2.3%) and for 0.1 µg., ± 0.0083 (coefficient of variation 8.6%). The esterase method was shown to be at least four times, and probably eight times more sensitive. The standard deviations of the two methods are of

the same order, although derived from different types of sample. The esterase method is, however, considerably simpler and quicker, and for this reason, as well as its greater sensitivity, further work aimed at improving the reproducibility seems justified.

CHAPTER 5

ENAMEL STUDIES

There are two main ways of studying the properties of enamel: in the intact tooth, or as a powder. The former has the advantage of being closer to the state in vivo, but requires sensitive methods since the surface area available is small. (It has been estimated that about 0.05 $\mu\text{g./sq.cm.}$ fluoride is taken up by an intact tooth surface²⁵). The surface area is increased by pulverising, but problems then arise in separating the enamel from other hard tissues.

Intact enamel

Most of the work on intact enamel has been concerned with solubility~~work~~, but isotopes have also been used¹⁶⁹. Attempts to use these methods in the mouth have been very limited. The first attempt was made by confining the liquid round an individual tooth with rubber dam⁴¹, and this

method was used to measure fluoride uptake from solutions containing 10 p.p.m. fluoride⁶. Limitation of access by adjacent teeth and the unknown effect of occlusal fissures are disadvantages of the method. A refinement of this technique has been suggested more recently⁴⁹, but without overcoming the disadvantages.

It is clearly desirable to confine the applied liquid to a defined part of the tooth surface. For work in vitro this is readily done with wax³¹; in the mouth a short cylinder of silicone rubber tubing has been used⁸⁶ for following pH changes. This technique has much to commend it where measurements can be completed in the mouth, although it would appear to be difficult to control the amount of stirring, which will have a marked effect on the rate of reaction with the enamel surface. In all these experiments the ratio of volume to exposed surface area is large, resulting in a high dilution of the products of the reaction. For many purposes this dilution is necessary in any case, since a certain minimum volume is required by the measuring instrument. On the other hand, it is also useful to be able to carry out a

reaction on the tooth surface, and it is then important to maintain the concentration of the substance measured as high as possible, by the use of the minimum volume possible. This was first achieved by Walter¹⁸³, who suggested the use of a tissue paper disc: the disc was impregnated with Methyl Violet and hydrochloric acid, and the time for a standard colour change as the enamel dissolved was recorded. In a study of the reliability of the method¹²⁶ using analysis of variance, and with filter paper replacing tissue paper, the standard deviation of the error of the technique was ± 16 seconds (coefficient of variation 10.6%) compared with the population standard deviation of ± 11 seconds. It is clear that no information can be obtained concerning individual teeth. (The enamel surface has a lower solubility than the interior⁹⁰, so that successive measurements on the same tooth, in addition to removing excessive amounts of enamel, could not be expected to improve the method very much).

The lack of reproducibility of this method appears to be due to the use of a visual assessment of colour. No commercial instruments

have been found capable of measuring this colour change, so that it is necessary to remove the disc and elute the products of the reaction. This procedure has the advantage of diluting the reagents, decreasing interference from them, but the product to be measured is also diluted. In addition to this a certain amount of liquid always remains on the tooth, and some means of measuring this loss must be available.

Since one of the most sensitive and accurate estimations is the molybdate method for phosphorus, a preliminary experiment was carried out to assess the practicability of this type of measurement (see Appendix G). Certain precautions are necessary, such as adequate cleaning of the tooth surface, and covering the filter paper with polythene, and the procedure occupies a good deal of time, but it appears to be practicable; no estimate of the contribution of technique to the standard deviation has so far been possible.

A disadvantage of these techniques is that they are difficult to adapt to curved surfaces. The surface tension of the solution is insufficient to overcome the stiffness of filter

paper and softer materials are not suitable for analytical procedures. Measurements are therefore limited at present to the labial surface of upper incisors.

It is of interest to consider whether the stage has been reached at which the fluoride content (or rather, the fluoride/phosphorus ratio) of surface enamel in the mouth can be measured. The enzymic method for fluoride requires about $0.05 \mu\text{g.}$ as a minimum; the enamel surface contains 0.1% fluoride approximately⁹¹; the density of enamel is 2.9 (discussed later); and the maximum practical diameter of the discs is 5 mm. These figures lead to an enamel thickness of just under 1μ which must be dissolved. Since the total thickness of enamel averages 1 mm. , this amount is close to the maximum which could justifiably be removed as a regular procedure. The measurement is therefore possible in theory, but it is still necessary to measure the phosphate present for comparison, and to ensure that no other components of enamel affect the enzyme and that none of the fluoride is reincorporated in the enamel. The latter could probably be achieved by dissolving the enamel with a strongly alkaline

solution of a chelating agent.

Powdered enamel

The classical method of obtaining enamel is to grind it off with a stone or bur. This has been useful where layers of enamel are required for comparison¹⁸⁹~~188~~, but it is necessary to stop before all the enamel has been removed to ensure that no dentine is included. The sample is then indubitably pure enamel, but not representative of whole enamel since the organic content of enamel rises towards the interior¹⁵⁴~~153~~.

Other methods of obtaining samples of enamel require some means for separating the enamel from other hard tissues. Heating to 150°C has been suggested¹⁵⁹~~158~~, causing the dentine to dry out and shrink away from the enamel, but the enamel is probably altered by this treatment. In 1935 Armstrong²⁹~~28~~ suggested using the procedure for mineral separation by immersion in a heavy liquid and later the use of a centrifuge was suggested by Manly and Hodge¹²³~~122~~, after whom the method is now known.

One or more teeth are dried, pulverised and placed in a mixture of bromoform and acetone of density 2.5 gm./ml.; enamel sinks to the bottom and dentine, cementum and pulp fragments float. The latter can be separated with a mixture of lower density if required. It becomes apparent, however, when this method is used in practice that not all the sample is sedimented in the bench centrifuges commonly recommended, and this is particularly noticeable when finely powdered material is used. Some investigators have avoided this issue by using a 60 or 100-mesh sieve fraction, which gives satisfactory results but provides a selected sample of enamel.

Another, more theoretical criticism is that the necessary drying may damage the organic material in the enamel and alter the characteristics of the pores. It is certainly desirable to compare dried with undried enamel powder. Methods for preparing undried enamel powder have been worked out, but it has been found to be a difficult material to use; there are difficulties in taking an adequately mixed sample from the stock, owing to differential

sedimentation of the particles, and difficulties in assessing how much enamel has been taken. Examination of the properties of enamel has so far only been carried out on enamel dried after separation.

Preparation of powder

Freshly extracted teeth of local origin were collected in tap water; on the same day, all the adherent soft tissue and any carious or discoloured tissue was removed under running water with burs. The surface of the enamel was also roughened slightly with a bur to remove any adherent organic film. The entire root was cut off, and the coronal pulp and about half the coronal dentine was removed, keeping the teeth wet all the time. The crowns of the teeth were then ground in an electric mortar at 4°C.

The grinding was at first carried out under water, with the aim of reducing local rise in temperature during crack formation (see Appendix B). It was found more convenient later to carry out the grinding under liquid

paraffin, and a slightly larger particle size appeared to result from this, although the grinding took longer. Light liquid paraffin B.P. was found most suitable. The powder ground under liquid paraffin was more easily dispersed in organic solvents than powder ground under water and this was important for adequate separation.

Separation of enamel powder

The density distribution of the powdered teeth was examined by successive centrifugations.

2 ml. of an aqueous suspension of powder was placed in a centrifuge tube, washed twice with acetone, and 6 ml. quantities of bromoform mixtures of increasing density were added. The suspension was shaken, centrifuged, and the supernatant was poured off, dried at 60°C. and weighed. The remaining sediment was shaken up with the next bromoform mixture and the procedure repeated.

The results are shown in Table XII. Two weaknesses in this procedure are apparent: each

Table XII over

T A B L E X I I

Density distribution of powdered teeth

Density gm./ml.	powder floating mg.	cumulative amount floating mg.
2.05	-	-
2.13	10	10
2.20	39	49
2.31	82	131
2.43	33	164
2.53	9	173
2.64	7	180
2.69	3	183
2.79	8	191
2.90	21	212
residue	103	315

[To face page 134]

liquid will be diluted slightly by the residue of the preceding liquid, so that the densities may be high by a few percent; and it is not possible to say whether the material floating was actually lighter than the liquid, or was so fine that it sedimented too slowly. No centrifuge was available to test this last point by using a higher speed; however the results indicate the separation which could be attained with the available equipment.

The enamel sediments to a large extent above 2.9 gm./ml. The dentine shows a wider spread with a peak at 2.2 - 2.31 gm./ml.; the increments of density differ in size, but this does not affect the qualitative conclusion. The most effective density for preparing enamel powder was selected as 2.85 gm./ml.

The density of dentine found here is higher than the value found by Manly and Hodge¹²³, who obtained a peak density of 2.13 - 2.16 gm./ml. This could well be due to the presence of water in the dentinal tubules rather than air which remains in the tubules when dried dentine is immersed in a liquid. This was checked by determining the density of pieces of dentine.

Intact pieces of root dentine (with cementum ground off) which had been stored in distilled water after cleaning, were weighed after blotting on filter paper and then attached to a thin piece of wire and weighed submerged in water. The wire was then weighed alone, with the end still in the water. The pieces of dentine were then dried to constant weight, first at room temperature and then at 105°C.

It is desired to calculate from these results the density of dentine with air filling the tubules and the density when water fills the tubules. Clearly the volume is the same in each case, but the weights differ by the amount of water lost. Since there is a considerable amount of water bound to the solid of dentine, it is assumed that low temperature drying removes only water in the tubules by evaporation, and that the density of the solid matter can be derived from the oven-dried weight and the volume after drying at room temperature. These assumptions are somewhat speculative but serve for the purpose of comparison.

The results are shown in Table XIII. There is considerable difference between the two

Table XIII over

TABLE XIII

Densities of dentine

	Weights in mg.	
	Specimen 1	Specimen 2
Wet in air	82.0	316.0
In water	46.1	166.6
Dried at room temperature	78.4	309.2
Dried at 105°C.	70.0	279.8

Densities in gm./ml.

	Specimen 1	
air in tubules	$\frac{78.4}{82.0 - 46.1}$	= 2.18
water in tubules	$\frac{82.0}{82.0 - 46.1}$	= 2.28
solid only	$\frac{70.0}{78.4 - 46.1}$	= 2.21

	Specimen 2	
air in tubules	$\frac{309.2}{316.0 - 166.6}$	= 2.07
water in tubules	$\frac{316.0}{316.0 - 166.6}$	= 2.12
solid only	$\frac{279.8}{309.2 - 166.6}$	= 1.96

[To face page 136]

specimens which could be due to differences in the degree of calcification, or possibly to a change in the surface tension on the wire between weighings. Nevertheless, it is clear that an appreciable change in the density of dentine occurs when the water in the tubules is replaced by air.

A difficulty occurred when the bromoform used in the separation procedure was recovered by redistilling: this appeared to remove some stabilising agent and an acid reaction developed. This was readily shown by shaking the bromoform with boiled water and adding Methyl Red. Purified bromoform was therefore stored with solid potassium bicarbonate and extracted with bicarbonate solution, followed by water, immediately before use. Under these conditions, no acid was detectable for about half an hour, which sufficed for a single operation.

Enamel powder ground and stored under liquid paraffin was placed in 30 ml. tubes and centrifuged down. 24 ml. of a mixture of bromoform and liquid paraffin of specific gravity 2.85 was added, shaken thoroughly and centrifuged at 3,500 g for 10 min. The

supernatant was poured off and the sediment was centrifuged again in a fresh bromoform mixture. The final sediment was washed 3 times in acetone and dried at 37°C.

Examination of enamel powder

The standard method for estimating the purity of enamel powder is to examine the particles under the microscope while immersed in a mixture of liquids having a refractive index intermediate between those of enamel and dentine¹²³. A diffraction effect, the Becke line, can be seen to move in opposite directions for the two types of particle when the focus is altered. This works well with large particles, but with particles of a few microns diameter the effect is difficult to detect. Furthermore, with particles of this size one begins to get fractionation of the tissues themselves. Dentine contains a proportion of a component of high density²⁰, and enamel contains areas of low density²⁶. However, dentine contains roughly thirty times as much organic matter as enamel

which suggests that a method based on estimation of an organic component might provide a satisfactory method for measuring contamination of enamel with dentine.

A method of this kind is described in Appendix C, in which a modification of the periodic-Schiff reaction is used to stain the organic component and the particles are examined microscopically. The colour of the smaller particles was, however, difficult to observe and there were always a number of particles with a pale stain. It seemed that a more reliable, and also a quantitative method might be obtained by dissolving the enamel and measuring the dye colorimetrically.

1 ml. 2N hydrochloric acid was added to the stained powder, and immediately it had been completely dissolved (about 30 sec.) 4 ml. 2N sodium hydroxide containing 0.1 M EDTA was added and the solution was incubated at 60°C. to digest the protein. 4 ml. M citric acid was then added to buffer the solution and the optical density was measured at 548 mμ.

A sample of powdered teeth was separated by a single centrifuging in a mixture of specific

gravity 2.7 into dentine and enamel, and the ratio of the amounts of stain in these was used as an index of the differentiation achieved. The method of staining described in Appendix C gave a ratio of 4; by increasing the concentration of basic fuchsin to 0.5% and washing the stained particles in alcohol, the ratio was increased to 11.8. The amount of stain per mg. of powder decreased when the weight of powder was increased, and the ratio also decreased.

This investigation has not been taken any further so far, partly because the opportunity to compare different samples of enamel has not presented itself, and partly because the improvement obtained did not appear sufficiently promising to warrant further investigation. It is felt that a more specific measurement of a component present in large excess in dentine would provide a better estimate of contamination, such as, for example, hydroxyproline (ratio approximately $70\frac{14}{100}$, although the amount in enamel is still subject to some uncertainty).

Table XIV over

TABLE XIV

Effect of centrifuging on enamel suspension

- 100 mg. dried enamel powder was incubated at 40°C in 100 ml. 0.1 N tris buffer pH 7.5 for 4 days with continuous stirring. A crystal of thymol was added to prevent growth of micro-organisms.
- 10 min. after stopping the stirrer, portions of the liquid were poured off and centrifuged for 10 min., as indicated.
- 1 ml. supernatant, 1 ml. molybdate reagent (see Appendix G for composition) and 8 ml. water were mixed and placed in a boiling water bath for 15 min.
- The extinction was measured at 830 mμ.

Method	Average force x g	Phosphorus μg./ml.
A	1	10.22
B	100	4.62
B	1,000	4.89
B	2,000	6.36
C	2,000	3.15
C	8,000	3.13
C	32,000	3.11
C	130,000	3.19
C	130,000	3.04

- A: Not centrifuged; supernatant poured off
- B: Centrifuged in conical base tubes; supernatant poured off
- C: Centrifuged in round bottom tubes; supernatant pipetted off

Removal of enamel powder from suspensions

It was pointed out by Neuman¹⁰⁵ (and confirmed later using glycerol-ashed bone¹⁴⁹) that suspensions of hydroxyapatite contained a fraction which was difficult to separate from water by ordinary filtration or centrifugation; centrifugation at 80,000 g gave an effective separation. Millipore filters¹⁴⁰, and dialysis¹¹⁷ have been also used. Some workers have been unable to detect this fraction²⁷. Since enamel is composed to a large extent of hydroxyapatite it was necessary to check whether these precautions were required in dealing with enamel powder. It is clear that enamel powder prepared by sedimenting from bromoform could hardly contain such fine material at the start; but it was possible that it might be formed as a result of equilibration with water.

In order to test this, a suspension of enamel powder was equilibrated for four days, and then centrifuged at various speeds and the phosphorus content of the supernatant was measured. The results are shown in Table XIV. It was concluded that 2,000 g sedimented all the

enamel, and that it was essential to remove the supernatant with a pipette to avoid disturbing the sediment.

Uptake of fluoride

The uptake of fluoride by calcium phosphate was initially treated as a process of adsorption, and the results obtained over a concentration range of 1 p.p.m. to 1% fluoride with a 30 minute contact were fitted to a Freundlich adsorption isotherm¹⁸¹. The exponent for enamel was distinctly lower than for the other forms of calcium phosphate. It appears quite reasonable to consider the initial uptake as an adsorption, but since the investigation of Neuman¹³⁸ the process has usually been considered in terms of ion exchange. This does not lead to any simple relation for the kinetics of the process and in the only attempt made so far, Knappwost⁹⁵ has fitted a diffusion equation to the uptake by hydroxyapatite crystals.

Several of the important variables in the reaction were investigated in 1939 by Smith and

Davey¹⁶⁵~~168~~. Using only steamed bone, they were primarily interested in the technical aspects, and did not attempt any theoretical interpretation apart from suggesting that over the range 0.4 - 8 p.p.m. final fluoride concentration, the uptake after several days was more characteristic of solid solution formation than adsorption. They were unable to detect any effect of temperature on fluoride uptake over the range 7 - 70°C. The uptake was decreased progressively above pH 7, although not completely abolished at pH 10, the highest pH tested. They showed also that 0.2 N sodium hydroxide was capable of increasing considerably the amount of additional fluoride which could be taken up by bone previously treated with fluoride, indicating at least partial reversal of the reaction. The fluoride uptake was found to be decreased when the particle size of the bone powder was greater. Knappwost⁹⁵~~94~~ has suggested an exponential relation between uptake and surface area, without presenting his results, however.

The small effect of temperature on uptake has been confirmed¹¹³~~112~~ with several different

varieties of hydroxyapatite and is good evidence that the process is primarily physical: chemical processes generally have a large temperature coefficient. The effect of pH has been shown to be linearly related to the logarithm of the fluoride uptake over a limited range of pH and bicarbonate shows a similar relation¹³⁸.

Similar data for enamel are rather less extensive. No investigation of the effect of temperature has been found, but the uptake was shown to increase as the surface area of the enamel powder was increased¹¹⁴. The uptake decreased as the pH was increased from 4.5 to 8.5⁵³ and N potassium hydroxide was able to remove fluoride from enamel powder at about the same rate as the fluoride had been taken up¹¹⁵.

Although the time course of the uptake of fluoride by enamel powder has been illustrated by several workers no figures accurate enough for kinetic analysis are available: the best are those of Muhler⁸², which can be improved by replotting the graph on a transformed scale, but are too erratic for any firm conclusions to be drawn.

Measurements of the effect of varying the

fluoride concentration have been made repeatedly. In one of the more recent studies¹⁰³, the conclusion was that 'the findings are in keeping with the view that fluoride is removed from solutions by adsorption or by exchange with hydroxyl ions of the crystal lattice to form fluorapatite'. This is still a fair summary of the present position, the only later contribution⁹⁹ being a suggestion that the decrease in the calcium and phosphorus concentrations which occurs during fluoride uptake implies that fluorapatite is precipitated and that this is the main cause of the decrease in fluoride concentration. The same phenomenon had been used earlier as a measure of the variation in solubility of mixed hydroxy-fluorapatites¹⁴⁶.

It is apparent that although the qualitative aspects of fluoride uptake have been clarified, little systematic quantitative information is available. It was felt that the initial step in this study must be to acquire more information concerning the time-course of the reaction after which the effect of external variables could be examined with more confidence.

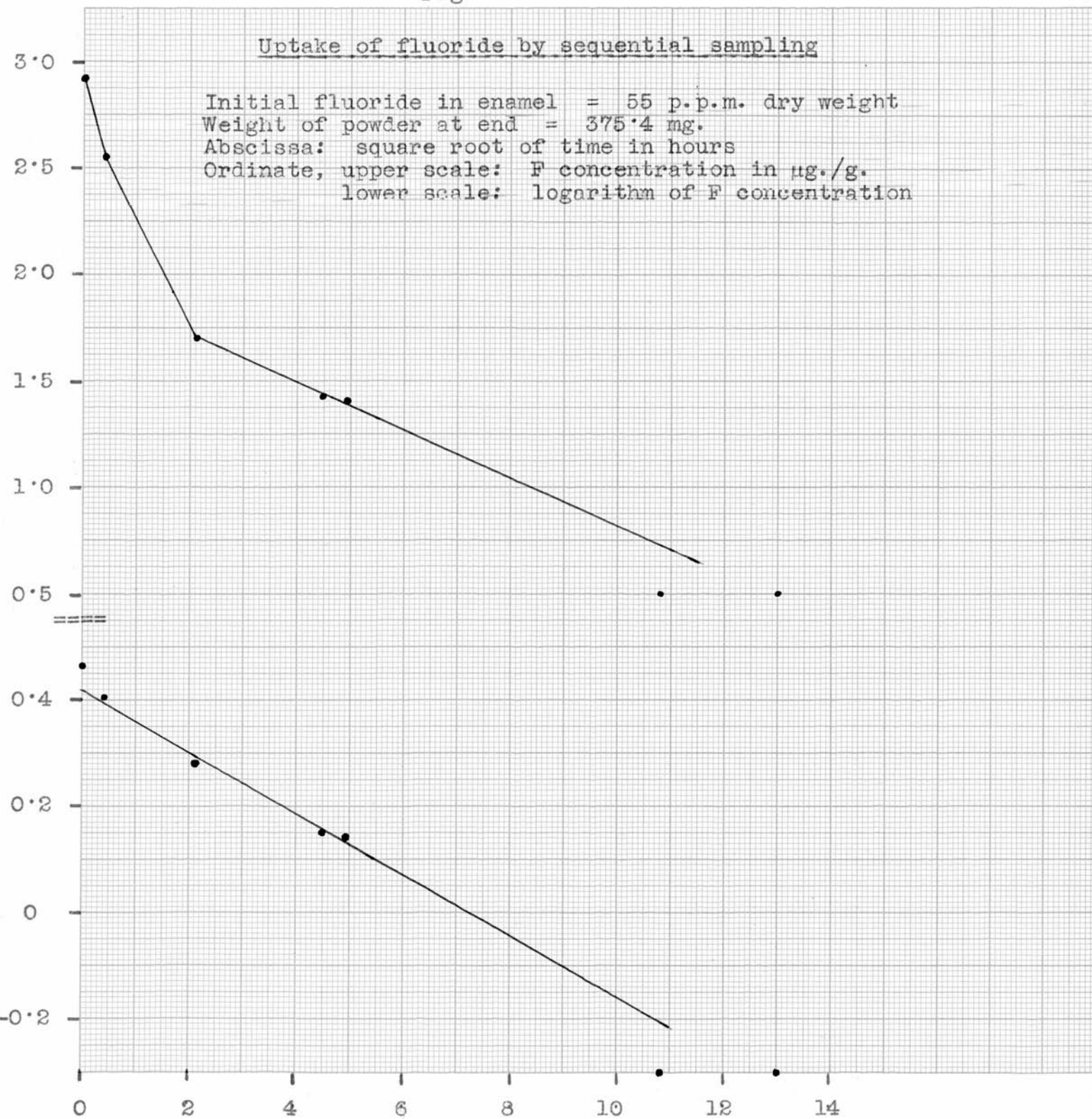
Time-course of fluoride uptake

Considerable importance was attached to the necessity for equilibrating the enamel powder with the solution before adding the fluoride: in all the previous studies mentioned (with a single exception⁹⁹) this point has been disregarded, complicating the interpretation by superimposing a hydration reaction and surface dissolution. It is difficult to eliminate the dissolution entirely (since the fluoride solution cannot be equilibrated) but it can be reduced considerably.

The first experiment was carried out by sequential sampling of a suspension:

Fresh enamel powder, prepared without drying, was added to 100 ml. 0.01 M phosphate buffer pH 7 in a polythene bottle and incubated at 25°C with magnetic stirring. After 20 hours 5 ml. NaF solution containing 60 µg./ml. F was added, and samples were removed at intervals through a Pt filter stick and polythene tubing into a diffusion bottle, which was weighed. After diffusion the fluoride content was measured polarographically. At the end of the experiment the powder was washed with benzene

Fig. 4



and weighed.

The results are shown in Fig. 4 (the theoretical treatment is developed later in the Chapter). The graph indicates the general course of the reaction: it should be noted that the accuracy of the last two points may have been affected by the necessity to delay the analysis for a period. Unfortunately, when this experiment was repeated on a more extensive scale, the sintered disc of the platinum filter stick was found to have completely disintegrated, presumably abraded by the enamel powder. In both experiments there was also a large amount of polythene powder present at the end, which was felt to be undesirable.

It was clear that the speed of stirring would have to be reduced considerably and the only convenient way of carrying this out was to rotate the whole container at an angle, removing the cap and inserting the sampling device for each sample.

When it is undesirable to remove any enamel powder from the container, the liquid can be sampled in two ways: by inserting a suitable filter connected to fine bore tubing, or by

centrifuging the container, sampling with a pipette and shaking up the powder again. The first method presents a number of technical difficulties, such as the removal of powder from the filter and the production of a suitable holder for the filter; these do not appear to be insuperable, but solving them might involve a lengthy investigation. Thus although the filter sampling method has the advantage of greater accuracy, it was decided to use smaller containers, which could be centrifuged and required less enamel, at least for the initial investigations.

Several experiments were carried out by this procedure, using the polarographic method for fluoride determination, but the results were too erratic to be useful, except as regards one point: after incubation for 2 - 3 days the fluoride in solution appeared to increase in some cases and it was found that the addition of a crystal of thymol prevented this increase, which was therefore assumed to be due to growth of micro-organisms.

The investigation was therefore postponed until the esterase method could be tested on the

Table XV over

TABLE XV

Effect of various substances on esterase activity

Method III

Added solutions adjusted to pH 6.5

Activities in arbitrary units corrected for blank; no. of measurements in brackets

Substance	Final concentration	Mean activity		Change %
		-	+	
Imidazole	0.02 M	0.352(2)	0.403(2)	+14.5
KHCO ₃	0.02 M	0.352(2)	0.316(2)	-10.2
Amprop.	0.02 M	0.352(2)	0.344(2)	- 2.3
Piperazine	0.02 M	0.352(2)	0.329(3)	- 6.5
	0.031 M	0.343(2)	0.286(1)	-12.5
Citrate	0.00667 M	0.343(2)	0.351(1)	+ 2.3
	0.02 M	0.343(2)	0.331(1)	- 3.5
Calcium	0.005 M	0.342(4)	0.321(1)	- 6.1
P-T buffer	diluted 6x	0.308(4)	0.311(2)	+ 1.0
Formalin *	0.1%	0.415(2)	0.611(2)	+47.2
Thymol *	33% saturated	0.415(2)	0.158(2)	-62.0

+ = Activity with substance added

- = Activity without substance added

Amprop. = 2-amino-2-methylpropanol

Composition of P-T buffer:

0.02 M Piperazine
 0.02 M Tris
 0.002 M Potassium dihydrogen phosphate
 0.24 N Hydrochloric acid
 Adjusted to pH 6.5 with potassium hydroxide

* All measurements in P-T buffer as above,
 except for 0.05 N perchloric acid in place
 of hydrochloric acid

[To face page 148]

system. When the esterase method had been developed, the effect on the enzyme of various possible components of the system was tested (see Table XV). A number of possible buffers were tested: imidazole clearly increased the activity, and has in fact been shown to catalyse ester hydrolysis itself³²; bicarbonate was inhibitory, but could probably be used at lower concentrations; aminomethylpropanol had no effect and piperazine was slightly inhibitory. However when piperazine was included in a suitable buffer, giving a lower final concentration, no inhibition was apparent. Of two possible antiseptics, thymol was strongly inhibitory and formalin appeared to have an activating effect. Citrate and calcium, as anticipated components of a solution in equilibrium with enamel, were also tested: citrate had no effect, calcium gave a doubtful inhibition. The next step was therefore to examine the effect of enamel supernatant:

10 mg. enamel was incubated with 10 ml. P-T buffer (see Table XV) for 2 days at room temperature and then centrifuged. 1 ml. portions of supernatant were treated by Method III, except that the enzyme was contained

in 0.7 ml., and 0.3 ml. water or 1 p.p.m. F solution was added.

The results were as follows, the figures in brackets being the number of measurements:

Activities (arbitrary units)	No F	With F
P-T buffer alone	0.287(1)	0.112(2)
Enamel supernatant	0.293(2)	0.111(4)

The standard deviation of the six measurements in the presence of fluoride was ± 0.003 . It was concluded that the enamel supernatant has no effect on the enzyme activity.

At this stage it was noted that Ericsson had claimed⁵⁴₅₅ that chloride inhibited fluoride uptake by enamel powder but did not inhibit uptake by hydroxyapatite or intact enamel: the enamel used was dried and the incubation period was 30 minutes. It is probable that the effect is connected with the rehydration of the enamel particles rather than the fluoride uptake, but in order to avoid a possible interference with the reaction, the P-T buffer, in which the enamel was to be incubated was made up with 0.05 N perchloric acid in place of 0.24 N hydrochloric acid. No

Table XVI over

T A B L E XVI

Initial fluoride uptake

Tube no.	Enamel mg.	Time mins.	Fluoride $\mu\text{g.}/\text{gm.}$
2	8.0	9.5	0.409
		31.5	0.383
		76	0.431
3	9.1	10.5	0.385
		32.5	0.417
		77	0.396
6	10.0	316	0.320
5	10.3	171	0.375
4	12.8	25.5	0.345
		66	0.329
1	14.2	137	0.350
		315	0.215 0.265

Regression of fluoride concentration on
weight of enamel gives:

Slope = -0.0124

Intercept = 0.507 $\mu\text{g.}/\text{gm.}$

Standard error
of intercept = ± 0.027

[To face page 150

specific check with this solution has been made, but the amount added to the enzyme solution was always constant, so that any systematic error would be eliminated. A short term experiment was carried out to examine the initial reaction of fluoride with enamel.

Weighed amounts of enamel powder were shaken with 9 ml. P-T buffer pH 6.6 in polythene test tubes and rotated at 1.5 r.p.m. at room temperature (18°C approximately) for 16 hours. 1 ml. 5 µg./ml. fluoride in P-T buffer was added to each tube and the rotation continued. At intervals the tubes were centrifuged and a sample of supernatant was removed with a polypropylene pipette and weighed. P-T buffer was added to make the volume to 1 ml. and the fluoride was determined by Method III.

The results are shown in Table XVI. There is no apparent shift in the fluoride concentration attributable to repeated sampling, and no change in the concentration is detectable in the interval 9.5 - 171 minutes, although a clear decrease occurs after 5.25 hours. There is a decrease in the fluoride concentration with

increasing amounts of enamel. It is to be expected that the decrease should be proportional to the amount of enamel. In order to test this the regression of fluoride concentration on weight of enamel was calculated excluding the last two samples taken. The intercept is very close to the initial fluoride concentration. The standard error is rather large owing to the long extrapolation required, but if any curvature had been present, the intercept would be correspondingly further away from the initial concentration. The initial uptake is therefore proportional to the amount of enamel added. The slope gives the uptake at the mean time (64 min.) and should be corrected to zero time for comparative purposes. In order to proceed further it was necessary to find some means of suppressing the growth of micro-organisms. Thymol, which had been found satisfactory in conjunction with polarographic measurements, could not be used as it inhibited the enzyme (Table XV). Chloroform was unsuitable as it would become mixed with the enamel powder. Toluene was next considered, but appeared to be unsatisfactory, since a layer on the surface

would interfere with the sampling. The ideal material would be solid, inert and without any ultraviolet absorption in the region used: no such material has been found so far. The use of toluene was therefore reconsidered. One possibility was to place the end of a fine sampling tube under the liquid: it would however result in complicating the apparatus considerably (because of the rotation and the necessity to seal the end). Another possibility, due to Dr. A.P. Ryle, was to include the toluene in a dialysis bag. A trial of this showed that overnight incubation was sufficient to saturate the buffer, as judged by ultraviolet absorption, and no film of toluene was visible on the surface of the buffer in a closed container. A longer experiment was therefore set up.

Weighed amounts of dried enamel powder were placed in 5 polythene tubes and shaken with 10 ml. P-T buffer. Several other tubes were also filled with 10 ml. P-T buffer to provide standards. A small dialysis sac containing toluene was placed in each tube and also in the remaining P-T buffer. The tubes were stoppered and incubated at $25 \pm 0.2^{\circ}\text{C}$. in

a water bath with rotation at 1.5 r.p.m. After 2 days, 1 ml. 5.5 μ g./ml. fluoride solution was added to all the tubes except one containing enamel, to which buffer was added. At intervals duplicate samples were removed with polypropylene pipettes after centrifuging and the tubes shaken up again. The samples were weighed and made up to 1 ml. with buffer, and the fluoride was estimated by Method III.

The experimental technique was designed to ensure that the toluene and buffer concentrations during the enzyme incubation were always constant and that the standards were kept under the same conditions as the experimental tubes. Nevertheless one difficulty in interpretation arose: the enzyme incubated with the sample from the tube containing enamel but no fluoride showed in the second estimation a higher activity, and in the fourth estimation a lower activity than the buffer alone, although there was good agreement in the first and third estimations. The agreement between the duplicates was good, however, so the difference was used to correct the activities in the other tubes containing enamel supernatant before calculating the fluoride concentration.

Table XVII over

T A B L E XVII

Fluoride uptake over longer period

Tube no.	Enamel mg.	Time hr.	Liquid removed gm.	Mean F $\mu\text{g./gm.}$ *
2	6.3	2.9	1.958	0.497
		26.0	0.993	0.385
		169.0	0.963	0.142
		361.2	1.940	0.061
4	9.7	2.9	2.004	0.473
		26.0	0.992	0.355
		169.0	1.122	0.128
		361.2	1.932	0.046
5	13.6	2.9	2.012	0.412
		26.0	1.001	0.275
		169.0	0.965	0.045
		361.2	1.858	0.009
3	20.0	2.9	1.977	0.353
		26.0	0.972	0.175
		169.0	0.970	0.017
		361.2	1.855	0.016

Initial volume = 11 ml.

Initial F concentration = 0.5 $\mu\text{g./ml.}$

* Mean measured fluoride concentration in liquid samples

The results are set out in Table XVII.

Theory of fluoride uptake

There is no general diffusion equation to describe data such as this, and the equation which describes the particular circumstances used⁴⁰ is not only too complicated for practical purposes, but makes several assumptions; it is difficult to assess whether these are justified or not. The situation is simplified by the fact that initially all diffusion equations show a close proportionality between the amount taken up and the square root of time. This relation holds true until the limits of the system begin to affect the diffusion. These limits are the thickness of the layer through which diffusion is occurring, the approach to equilibrium, and the depletion of the source of diffusible material. In the present case, no information is available on the maximum diffusion pathway, and it must be assumed not to be limiting; and no evidence of equilibrium has been found. The source, however, is clearly limiting if the

fluoride concentration falls considerably, as it must if the changes are to be measured.

We have therefore:

$$(F_0 - F)V \propto m\sqrt{t} \quad \text{for diffusion}$$

where F = fluoride concentration
 F_0 = initial fluoride concentration
 V = volume of solution
 m = weight of enamel
 t = time

$$\text{Therefore } \frac{dF}{dt} \propto \frac{2m}{V\sqrt{t}}$$

As an approximation to the effect of decreasing fluoride concentration we put

$$\frac{dF}{dt} \propto F$$

Combining these two relations gives

$$\frac{dF}{dt} = k F \frac{2m}{V\sqrt{t}}$$

Integrating gives

$$\ln F = k \frac{m}{V} \sqrt{t} + \ln (1 - am)F_0V_0$$

where V_0 = initial volume
 a = initial fluoride uptake per unit weight of enamel and per unit concentration of fluoride

The value of V used here must clearly be the mean volume weighted for the corresponding time interval, when the same solution is sampled

Fig. 5

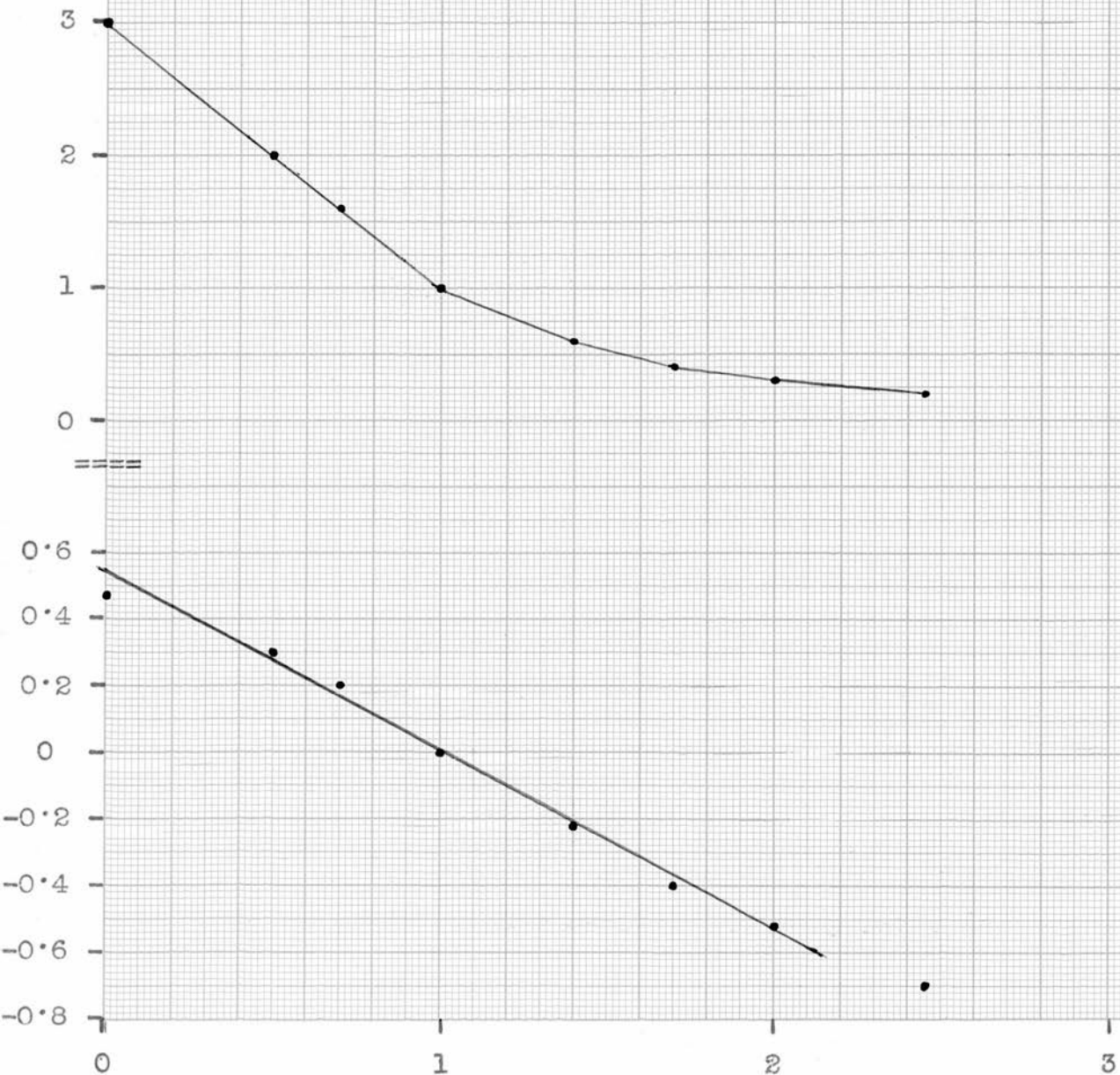
Fluoride uptake by steamed bone

replotted from Smith and Davey ¹⁶⁵

Abscissa: square root of time in hours

Ordinate, upper scale: F concentration in $\mu\text{g./ml.}$

lower scale: logarithm of F concentration



repeatedly. In view of the numerous approximations, this equation cannot be expected to give an accurate fit, but as a possible means of clarifying an otherwise rather intractable problem, it could be useful.

The only data in the literature¹⁶⁵ ~~168~~ which can be used to check the equation are shown in Fig. 5 replotted from the original graph, which showed the change in the fluoride concentration of a solution in contact with steamed bone. The volume changes were presumably negligible. When the fluoride concentration is plotted against the square root of time, a straight line is obtained until slightly over half the fluoride has been taken up, after which a marked curvature appears. On the other hand when the logarithm of the fluoride concentration is used the line is virtually straight up to one tenth of the original concentration, beyond which the experimental error probably becomes rather large. The logarithmic plot gives an intercept greater than the original concentration, but it is not clear whether this should be attributed to deviation from theory or to experimental error.

The same type of graph has been used in

Fig. 6

Fluoride uptake over longer period

Data from Table XVI

Abscissa: square root of time x concentration of enamel

Ordinate: logarithm of fluoride concentration

Lines link samples from same tube (no. in brackets)

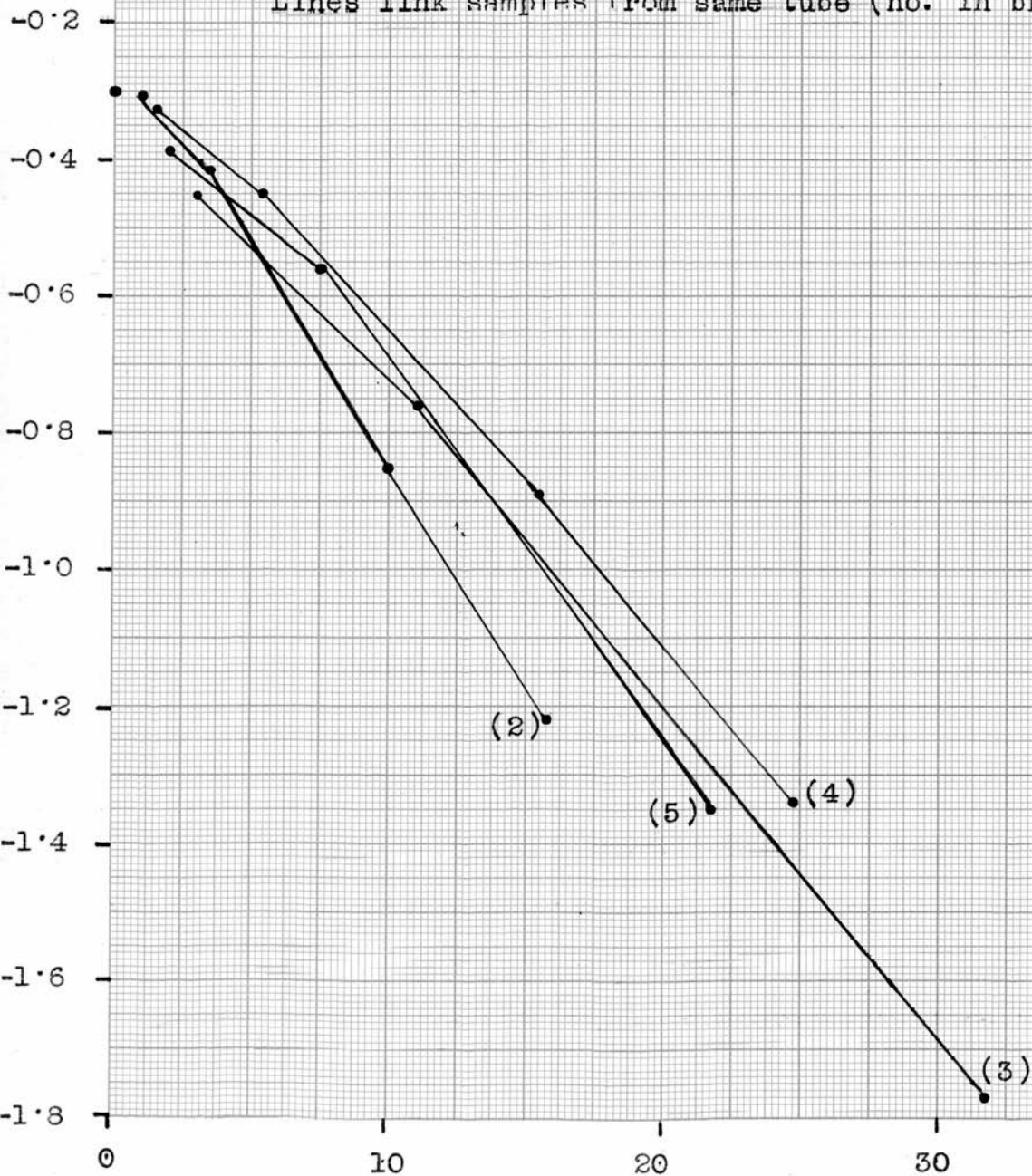


Fig. 4, and the same features are apparent, although the scatter of the points is rather wider. The maximum volume change here was less than 3% of the total volume.

An attempt can now be made to interpret the data in Table XVII. The logarithmic plot is used in Fig. 6 since the experiment is mainly concerned with the later stages of the reaction. The general form is similar to that of Fig. 4, showing that the volume correction is useful, and the lines have been brought considerably closer by the correction for the amount of enamel. There is still some difference in the slopes of the lines, but it is not related to the amount of enamel; it is suggestive of a systematic difference between the tubes rather than erratic fluoride measurements. Nevertheless the agreement between duplicates in this experiment was not as good as it could have been and an improvement here would greatly assist in the interpretation.

Discussion

The preparation of enamel powder involves several procedures which could affect its properties. The only satisfactory method of testing this is to examine the properties after varying the preparation procedure, and this cannot be carried out usefully until the properties themselves have been investigated thoroughly. Thus choosing a method of preparation for enamel is largely a matter of intuition at present, unsatisfactory as this may be.

In the only part of the preparation which could be evaluated independently, namely the separation of enamel and dentine, the evaluation is limited by the lack of suitable tests, mainly owing to the correlation between the methods of testing and separating the two tissues. The density is correlated with the refractive index¹²²~~123~~ and with the proportion of organic matter; and virtually all the minor components are correlated either with the organic or the inorganic component. Other properties e.g. absorption of X-rays and fluorescence¹¹²~~122~~, tend to be correlated

in the same way.

It can be said that large particles of enamel and dentine can be separated and recognised readily, but that small particles present difficulties in both procedures. Unfortunately large particles are difficult to produce in quantity, and are usually associated with a proportion of dust anyway. In these circumstances one is forced to prepare mixed batches of enamel of somewhat doubtful purity and postpone evaluation of the manipulative procedures.

It has been shown that certain data on fluoride uptake in the literature and the results of initial experiments on enamel powder recorded here can be described in terms of a diffusion process. There is an initial rapid uptake which occurs within a few minutes and then a slower diffusion process which appears to extend over a period of weeks. It is probable that the initial uptake occurs on the external surface of the enamel particle. In the next stage one would have expected to find a further uptake over a few hours by the surfaces of the crystals inside the particle followed by a slow uptake into

the interior of the crystals. The fact that there appears to be only a single diffusion process suggests that the internal surfaces may take up fluoride by a process different from that occurring on the external surfaces which have been exposed by fracture. This would imply, as knowledge of the structure of hard tissues also implies¹⁰⁴~~that~~, that there is a continuous chemical bonding between the inorganic and organic components.

CHAPTER 6

CONCLUSION

This record began with caries and ended with caries, but the work described represents only the start of the investigation. Two ultimate objectives have directed the nature of the investigation: the need for data on the process of incorporation of fluorine during the formation of enamel, and on the ways in which the incorporated fluorine might react during carious destruction of enamel; and the need for means of establishing the relative importance of the various factors which influence the occurrence and progress of caries.

The initiation of this research has involved the acquisition and testing of a number of techniques, several of which are associated more with industrial research. Some of these have been found useful, some have been discarded, and others have been left for more detailed consideration in the future.

The results of the work have been the partial characterisation of an enzyme which is inhibited

by fluoride well below 0.1 p.p.m.; the use of this enzyme for analytical purposes; the recognition of the importance of diffusion processes in the reaction between fluoride and enamel; and the correlation of some visible features of teeth with the presence of caries. Other results which have already been published are referred to in the appropriate section.

The characterisation of an enzyme inhibited by low levels of fluoride is of interest from the point of view of the toxicity of fluoride, since Armstrong¹⁶⁷~~180~~ has concluded that no human enzymes are inhibited appreciably by 1 p.p.m. fluoride. It is unfortunate that it is not yet possible to decide whether the inhibition of the esterase described here is competitive or noncompetitive and no conclusion is possible concerning the effect of fluoride on the function of the enzyme in the cell. Nevertheless the enzyme has been found useful as an analytical tool, and the actual measurement of fluoride is the simplest and quickest, as well as the most sensitive available, which makes possible experiments requiring a considerable number of determinations. The only comparable method⁶¹~~63~~ which uses reverse

isotope dilution and adsorption of ^{18}F , is stated to have an uncertainty of 20%, but is much more specific⁹⁷.

Diffusion has long been recognised as a part of any reaction between enamel and substances in solution, but the only work in which it is implicated as a limiting factor is concerned with the dissolution of enamel by acids⁷³; the time-course is, however, interpreted in terms of accumulation of reaction products. Limitation by diffusion makes the assessment of any associated chemical reactions rather difficult. On the other hand it is possible that the basic reaction could be explained by activated diffusion, which might simplify the interpretation.

CHAPTER 7

SUMMARY

The principal stages in the development of knowledge of fluorine, particularly in relation to dental tissues have been surveyed and the present knowledge of the relation between fluorine and dental caries has been indicated. Evidence available in the literature concerning the structure of dental enamel and its reaction with fluoride has been described, and various methods for measuring fluoride have been outlined.

A polarographic method for fluoride estimation has been investigated and modifications have been suggested. A new polarographic method for fluoride estimation has been proposed. A method for separating fluoride by diffusion prior to estimation has been tested.

The partial purification of a new enzyme from liver is described and a new substrate for the determination of its activity has been used. The kinetics of the enzyme and the characteristics of its inhibition by fluoride have been examined and a method for estimating fluoride using this

inhibition has been studied.

Methods for the preparation of enamel powder have been described and the kinetics of the reaction between enamel powder and fluoride have been investigated. The solubility of intact enamel in the mouth has been investigated.

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Abbreviations used

°	°
Å.	Ångström unit
ADP	adenosine diphosphate
ATP	adenosine triphosphate
DMF	total number of decayed, missing and filled teeth
EDTA	1,2-ethylene-di(nitrilo-diacetic acid)
M	molar concentration
meq.	milliequivalents
N	equivalent concentration
n	degrees of freedom
NADH	reduced nicotinamide adenine dinucleotide
p.p.m.	parts per million
P-T buffer	see Table XV
t	ratio of mean to standard error
tris	2-amino-2-hydroxymethyl-1,3-propandiol
V.	volts
μamp.	microamperes

CHAPTER 8

REFERENCES

Reports

1. COMMISSION on ENZYMES of International Union of Biochemistry: Report. Pergamon Press, Oxford, 1961
2. EXPERT COMMITTEE on Dental Health: Standardisation of reporting of dental diseases. W.H.O. Tech. Rep. Ser., 242, 1 - 23 (1962)
3. MINISTRY of HEALTH, Scottish Office and Ministry of Housing and Local Government: The conduct of the fluoridation studies in the United Kingdom and the results achieved after five years. Rep. Publ. Hlth Med. Subj., No. 105, 1 - 50 (1962)

Authors

4. A.J. ADLER and G.B. Kistiakowsky: Isolation and properties of pig liver esterase. J. biol. Chem., 236, 3240 - 3245 (1961)
5. A.J. ADLER and G.B. Kistiakowsky: Kinetics of pig liver esterase catalysis. J. Amer. chem. Soc., 84, 695 - 703 (1962)
6. P. ADLER and J. Straub: Uptake of fluorine intravitaly by intact human teeth from a topically applied sodium fluoride solution. Experientia, 6, 24 - 25 (1950)

7. P. ADLER: The connections between dental caries experience and water-borne fluorides in a population with low caries incidence. J. dent. Res., 30, 368 - 381 (1951)
8. N.J. AINSWORTH: Mottled teeth. Brit. dent. J., 55, 233 - 250 (1933)
9. S. AMBERG and A.S. Loevenhart: Further observations on the inhibiting effect of fluorides on the action of lipase together with a method for the detection of fluorides in food products. J. biol. Chem., 4, 149 - 164 (1908)
10. D.B. AST, D.J. Smith, B. Wachs and K.T. Cantwell: Newburgh-Kingston caries-fluorine study. XIV. Combined clinical and roentgenographic dental findings after ten years of fluoride experience. J. Amer. dent. Ass., 52, 314 - 325 (1956)
11. H.F. ATKINSON: An investigation into the permeability of enamel using osmotic methods. Brit. dent. J., 83, 205 - 214 (1947)
12. O. BACKER-DIRKS, B. Houwink and G.W. Kwant: Some special features of the caries-preventive effect of water fluoridation. Arch. oral Biol., 4, 187 - 192 (1961)
13. A.K. BALLS and H.N. Wood: Acetyl chymotrypsin and its reaction with ethanol. J. biol. Chem., 219, 245 - 256 (1956)
14. G.C. BATTISTONE and G.W. Burnett: Studies of the composition of teeth. V. Variations in the amino acid composition of dentin and enamel. J. dent. Res., 35, 263- 272 (1956)
15. C.A. BEEVERS and D.B. McIntyre: The atomic structure of fluor-apatite and its relation to that of tooth and bone mineral. Min. Mag., 27, 254- 257 (1946)
16. R. BELCHER, M.A. Leonard and T.S. West: The preparation and analytical properties of NN-di(carboxymethyl)aminomethyl derivatives of some hydroxyanthraquinones. J. chem. Soc., 160, 2390 - 2393 (1958)

17. R. BELCHER, M.A. Leonard and T.S. West:
A new spot test for the detection of
fluoride ions. Talanta, 2, 92 - 93 (1959)
18. R. BELCHER and T.S. West: A comparative
study of some lanthanon chelates of alizarin
complexan as reagents for fluoride.
Talanta, 8, 863 - 870 (1961)
19. F. BERGMAN and S. Rimon: The effect of pH
variations on the activities of C-esterase.
Biochem. J., 70, 339 - 344 (1958)
20. G. BERGMAN and B. Engfeldt: Studies on
mineralized dental tissues with special
reference to the dentinal tubules.
Acta odont. scand., 13, 1 - 7 (1955)
21. G. BERGMAN: Microscopic demonstration of
liquid flow through human dental enamel.
Arch. oral Biol., 8, 233 - 234 (1963)
22. S.A. BERNHARD, W.C. Coles and J.F. Nowell:
Kinetics of the system α -chymotrypsin methyl
hippurate water hydroxylamine: the role of
water in enzymatic hydrolysis. J. Amer.
chem. Soc., 82, 3043 - 3050 (1960)
23. J.S. BEVERIDGE, B.J. MacNulty, G.F. Reynolds
and E.A. Terry: The polarographic
determination of fluoride. Part II. The
determination of fluorine in bromine,
hydrochloric acid and hydrobromic acid.
Analyst, 79, 267 - 272 (1954)
24. J. BJERRUM, G. Schwarzenbach and L.G. Sillén:
Stability constants of metal-ion complexes
and solubility products of inorganic
substances. Part II. Inorganic ligands.
Special Publication No. 7, Chemical Society,
London, 1958.
25. J.F. BONNER: Absorption du radio-fluor par
l'émail dentaire intact. Schweiz. Mschr.
Zahnheilk., 65, 652 (1955)
26. A.K. BOSE, R.Q. Blackwell and L.S. Fosdick:
Fractionation of human tooth enamel on the
basis of density. J. dent. Res., 39,
141-149 (1960)

27. M. BOULET and J.R. Marier: Precipitation of calcium phosphates from solutions at near physiological concentrations. Arch. Biochem. Biophys., 93, 157 - 165 (1961)
28. E.R. BRANSBY and J.R. Forrest: The dental condition of children in seven areas in England and Wales as shown by the base-line dental examinations made in connection with the fluoridation demonstration studies. Mon. Bull. Min. Hlth, 17, 28 - 34 (1958)
29. P.J. BREKHUS and W.D. Armstrong: A method for the separation of enamel, dentin and cementum. J. dent. Res., 15, 23 - 29 (1935)
30. W.E. BROWN: Octacalcium phosphate and hydroxyapatite: crystal structure of octacalcium phosphate. Nature, 196, 1048 - 1050 (1962)
31. F. BRUDEVOLD: A study of the phosphate solubility of the human enamel surface. J. dent. Res., 27, 320 - 329 (1948)
32. T.C. BRUCE and G.L. Schmir: Imidazole catalysis. I. The catalysis of the hydrolysis of phenyl acetates by imidazole. J. Amer. chem. Soc., 79, 1663 - 1667 (1957)
33. W. BÜTTNER, S. Schülke and S. Soyka: Eine einfache Bestimmung kleiner Mengen von Fluor 5 - 30 µg. in Knochen und Zahnhartgeweben durch Diffusion. Dtsch. zahnärztl. Z., 18, 24 - 28 (1963)
34. D. CARLSTRÖM: X-ray crystallographic studies on apatites and calcified structures. Acta Radiol., 43, Suppl. 121, 1 - 59 (1955)
35. D. CARLSTRÖM: Particle size and chemical composition of the crystallites in bone and synthetic apatites. Biochim. Biophys. Acta, 17, 603 - 604 (1955)
36. D. CARLSTRÖM and J.-E. Glas: The size and shape of the apatite crystallites in bone as determined from line-broadening measurements on oriented specimens. Biochim. Biophys. Acta, 35, 46 - 53 (1959)

37. H.V. CHURCHILL: Occurrence of fluorides in some waters of the United States.
Ind. Eng. Chem., 23, 996 - 998 (1931)
38. P.A. Clifford: Report on fluorine.
J. Ass. off. agr. Chem., 24, 350 - 363 (1941)
39. R. COLLINS, W. Nebergall and H. Langer:
A study of the reactions of various tin(II) compounds with calcium hydroxylapatite.
J. Amer. chem. Soc., 83, 3724 - 3725 (1961)
40. J. CRANK: The mathematics of diffusion.
Oxford University Press, London, 1956
41. G. CSOBÁN: Die Experimentalmethodik zur Prüfung der kariösen Entkalkung der Zahnhartsubstanzen. Ost. Z. Stomat., 46, 220 - 226 (1949)
42. A.I. DARLING, K.V. Mortimer, D.F.G. Poole and W.D. Ollis: Molecular sieve behaviour of normal and carious human enamel.
Arch. oral Biol., 5, 251 - 273 (1961)
43. G.N. DAVIES, R.M. King and A.A. Collins: Relationship between lactobacillus counts, Snyder tests and the subsequent incidence of dental caries. Arch. oral Biol., 1, 62 - 73 (1959)
44. H.M. DAVIS and J.E. Seaborn: A linear sweep cathode-ray polarograph. Electronic Eng., 25, 314 - 321 (1953)
45. H.T. DEAN: Endemic fluorosis and its relation to dental caries. U.S. Publ. Hlth Rep., 53, 1443 - 1452 (1938)
46. H.T. DEAN, F.A. Arnold Jr. and E. Elvove: Domestic water and dental caries.
V. Additional studies of the relation of fluoride domestic waters to dental caries experience in 4,425 white children aged 12 to 14 years of 13 cities in 4 states.
U.S. Publ. Hlth Rep., 57, 1155 - 1179 (1942)
47. M. DIXON and E.C. Webb: Enzymes. Longmans, Green and Co., London, 1958

48. R. DUFAIT and L. Massart: Le fluorure de sodium comme inhibiteur de réactions fermentaires. Enzymologia, 7, 337 - 338 (1939)
49. E.EIGEN, A.R. Volpe, F.M. Lapeyrolerie, S. Weiss and W.J. King: An in vivo technique for determining the effect of agents on enamel solubility in human subjects. Arch. oral Biol., 7, 459 - 460 (1962)
50. P.J. ELVING, C.A. Horton and H.H. Willard: Analytical chemistry of fluorine and fluorine containing compounds. Fluorine Chemistry, 2, 51 - 211 (1954)
51. H.R. ENGLANDER, W.J. Carter and L.S. Fosdick: The formation of lactic acid in dental plaques. III. Caries immune individuals. J. dent. Res., 35, 792 - 799 (1956)
52. ERHARDT: Kali fluoratum zur Erhaltung der Zähne. Memorabilien, 19, 359 (1874)
J. Amer. dent. Ass., 49, 385 (1954)
53. Y. ERICSSON: Fluorides in dentifrices; investigations using radioactive fluorine. Acta odont. scand., 19, 41 - 77 (1961)
54. Y. ERICSSON: Effect of chloride ions on the fluoride uptake by dental enamel. Acta odont. scand., 20, 379 - 392 (1962)
55. J.S. FALCONER and D.B. Taylor: The initial stages in the purification of pig liver esterase. Biochem. J., 40, 831 - 834 (1946)
56. H. FERNÁNDEZ-MORÁN and A. Engström: Electron microscopy and X-ray diffraction of bone. Biochim. Biophys. Acta., 23, 260 - 264 (1957)
57. D.J. FERRETT, G.W.C. Milner, H.I. Shalgosky and L.J. Slee: A comparative study of three recently developed polarographs. Analyst, 81, 506 - 512 (1956)
58. L. FLEISCHMAN: Ueber Kiefer-rachitis und deren Einfluss auf das Milchgebiss. Wiener med. Presse, 18, 516 - 520 (1877)

59. J.R. FORREST, J. Longwell, H.H. Stones and A.M. Thomson: The fluoridation of domestic water supplies in North America as a means of controlling dental caries. H.M. S.O., London, 1953
60. J. FORREST: Caries incidence and enamel defects in areas with different levels of fluoride in the drinking water. Brit. dent. J., 100, 195 - 200 (1956)
61. J.H. FREMLIN, J.L. Hardwick and J. Suthers: Measurement of small quantities of fluoride with the help of fluorine-18. Nature, 180, 1179 - 1181 (1957)
62. J.H. FREMLIN, J.L. Hardwick and J. Mathieson: The value of isotope studies of the uptake and release of small ions by the hard dental tissues. J. dent. belge, 50, 51 - 62 (1959)
63. F.J. FRERE: Microdiffusion method for the determination of fluorides. Microchem. J., 6, 167 - 170 (1962)
64. J.S. FRIEDENWALD and G.D. Maengwyn-Davies: Elementary kinetic theory of enzymatic activity: first order theory. The mechanism of enzyme action, ed. by W.D. McElroy and B. Glass, 154 - 179. Johns Hopkins Press, Baltimore, 1954
65. S. GABRIEL: Chemische untersuchungen über die Mineralstoffe der Knochen und Zähne. Z. physiol. Chem., 18, 257 - 303 (1894)
66. D.J. GALAGAN and J.R. Vermilion: Determining optimum fluoride concentrations. U.S. Publ. Hlth Rep., 72, 491 - 493 (1957)
67. I. GEDALIA, K.A. Rosenzweig and A. Sadeh: Fluorine content of superficial enamel layer and its correlation with the fluorine content of saliva. J. dent. Res., 40, 865 - 869 (1961)
68. F. GIESECKE and W. Rathje: Zur Kenntnis der phosphate: III. Hydroxyl-Fluor-apatite des Mineral der Rohphosphate. Ber. chem. Ges., 74B, 349 - 356 (1941)

69. C.W. GISH, J.C. Muhler and C.L. Howell:
New approach to the topical application of
fluorides for the reduction of dental caries
in children: results at the end of five
years. J. Dent. Child., 29, 65 - 71 (1962)
70. G. GOMORI: Human esterases. J. Lab. clin.
Med., 42, 445 - 453 (1953)
71. G. GOMORI and R.D. Chessick: Histochemical
studies of the inhibition of esterases.
J. cell. comp. Physiol., 41, 51 - 63 (1953)
72. R.M. GRAINGER and C.I. Coburn: Dental caries
of the first molars and the age of children
when first consuming naturally fluoridated
water. Can. J. publ. Hlth, 46, 347 - 354
(1955)
73. J.A. GRAY: Kinetics of dissolution of
human dental enamel in acid. J. dent. Res.,
41, 633 - 645 (1962)
74. P. GREENGARD: Determination of intermediary
metabolites by enzymic fluorimetry.
Nature, 178, 632 - 634 (1956)
75. L. GREENLAND: Separation of fluorine by
microdiffusion of hydrofluoric acid.
Anal. chim. Acta, 27, 386 - 389 (1962)
76. B.E. GUSTAFSSON, C.-E. Quensel, L.S. Lanke,
C. Lundqvist, H. Grahnen, B.E. Bonow and
B. Krasse: The Vipeholm dental caries
study: the effect of carbohydrate intake on
caries activity in 43 individuals observed
for five years. Acta odont. scand., 11,
232 - 364 (1954)
77. H. GUTFREUND and J.M. Sturtevant:
Mechanism of reaction of chymotrypsin with
p-nitrophenyl acetate. Biochem. J., 63,
656 - 661 (1956)
78. R.J. HALL: Determination of microgram
amounts of fluorine by diffusion. Analyst,
85, 560 - 563 (1960)

79. R.J. HALL: The spectrophotometric determination of sub-microgram amounts of fluorine in biological material. Analyst, 88, 76 - 83 (1963)
80. J.L. HARDWICK and J.H. Fremlin: Isotope studies on the permeability of the dental enamel to small particles and ions. Proc. roy. Soc. Med., 52, 752 - 758 (1959)
81. J.M. HARVEY: Chronic endemic fluorosis of Merino sheep in Queensland. Queensland J. agr. Sci., 9, 47 - 141 (1952)
82. W.E. HATTON, W.H. Nebergall and J.C. Muhler: The removal of fluorine from dilute solutions of sodium fluoride and stannous fluoride by powdered dental enamel. J. dent. Res., 34, 350 - 357 (1955)
83. R.L. HAYES, N.W. Littleton and C.L. White: Post-eruptive effect of fluoride on first permanent molars of children in Grand Rapids, Michigan. Amer. J. publ. Hlth, 47, 192 - 199 (1957)
84. W.B. HEALY, T.G. Ludwig and F.L. Losee: Soils and dental caries in Hawke's Bay, New Zealand. Soil Sci., 92, 359 - 366 (1961)
85. B.H.J. HOFSTEE: Spectrophotometric determination of esterases. Science, 114, 128 - 130 (1951)
86. A.W. HOLMES and J.D. Middleton: An in vivo estimation of enamel solubility. Brit. dent. J., 113, 380 - 386 (1962)
87. B.L. HORECKER and A. Kornberg: The extinction coefficients of the reduced band of pyridine nucleotides. J. biol. Chem., 175, 385 - 389 (1948)
88. C.R. HOSKINS and C.F.M. Fryd: The determination of fluorine in Piltown and related fossils. J. appl. Chem., 5, 85 - 87 (1955)
89. H. HUGUETTE and M.J. Dallemagne: Main mineral constituent of bone and teeth. Arch. oral Biol., 5, 137 - 144 (1961)

90. S. ISAAC, F. Brudevold, F.A. Smith and D.E. Gardner: Solubility rate and natural fluoride content of surface and subsurface enamel. J. dent. Res., 37, 254 - 263 (1958)
91. S. ISAAC, F. Brudevold, F.A. Smith and D.E. Gardner: The relation of fluoride in the drinking water to the distribution of fluoride in enamel. J. dent. Res., 37, 318 - 325 (1958)
92. D. JACKSON and S.M. Weidmann: Relationship between age and fluorine content of human dentine and enamel: a regional survey. Brit. dent. J., 107, 303 - 306 (1959)
93. G.N. JENKINS: Effect of pH on the fluoride inhibition of salivary acid production. Arch. oral Biol., 1, 33 - 41 (1959)
94. M.J. JOHNSON: A rapid micromethod for estimation of non-volatile organic matter. J. biol. Chem., 181, 707 - 711 (1949)
95. A. KNAPPWOST: Zur Kinetik des Fluor-Hydroxyl-Ionenaustausches am Hydroxylapatit. Naturwiss., 19, 555 - 556 (1959)
96. D.N. KRAMER and R.M. Gamson: Studies of the fine structure of the isomeric indophenyl esters: biochemical implications. J. biol. Chem., 235, 1785 - 1789 (1960)
97. J.N. KUDAH: On the determination of fluoride in small samples. Arch. oral Biol., Suppl., 53 - 56 (1962)
98. J.N. KUDAH, J.H. Fremlin and J.L. Hardwick: The isotope-dilution method of fluorine microestimation. Radioisotopes in the physical sciences and industry, 2, 317 - 323. International Atomic Energy Association, Vienna, 1962.
99. A.C. KUYPER and K. Kutnerian: Mechanism of incorporation of fluoride into bone salt. J. dent. Res., 41, 345 - 350 (1962)
100. F.M. KYES, N.J. Overton and T.W. McKean: Clinical trials of caries inhibitory dentifrices. J. Amer. dent. Ass., 63, 189 - 193 (1961)

101. K.J. LAIDLER: The chemical kinetics of enzyme action. Oxford University Press, London, 1958
102. A. LANGER: Amperometric titration of fluorine with thorium nitrate. Ind. Eng. Chem., Anal. Ed., 12, 511 - 514 (1940)
103. S.A. LEACH: Reactions of fluoride with powdered enamel and dentine. Report of a chemical study over a range of concentrations of sodium fluoride. Brit. dent. J., 106, 133 - 142 (1959)
104. A.G. LEAVER, J.E. Eastoe and R.L. Hartles: Citrate in mineralised tissues. II. The isolation from human dentine of a complex containing citric acid and a peptide. Arch. oral Biol., 2, 120 - 126 (1960)
105. G.J. LEVINSKAS and W.F. Neuman: The solubility of bone mineral. I. Solubility studies of synthetic hydroxylapatite. J. phys. Chem., 59, 164 - 168 (1955)
106. R.C. LIKINS, F.J. McClure and A.C. Steere: Urinary excretion of fluoride following defluoridation of a water supply. U.S. Publ. Hlth Rep., 71, 217 - 220 (1956)
107. H.W. LINDE: Estimation of small amounts of fluoride in body fluids. Anal. Chem., 31, 2092 - 2094 (1959)
108. H. LINEWEAVER and D. Burk: Determination of enzyme dissociation constants. J. Amer. chem. Soc., 56, 658 - 666 (1934)
109. K. LITTLE: Electron microscope studies on human dental enamel. J. roy. micr. Soc., 78, 58 - 66 (1959)
110. M.F. LITTLE, J. Posen and L. Singer: Chemical and physical properties of altered and sound enamel. III. Fluoride and sodium content. J. dent. Res., 41, 784 - 789 (1962)

111. A.S. LOEVENHART and G. Peirce: The inhibiting effect of sodium fluoride on the action of lipase. J. biol. Chem., 2, 397 - 413 (1907)
112. F.L. LOSEE, W.H. Jennings, M.E. Lawson Jr. and A.F. Forziati: Microstructure of the human tooth. J. dent. Res., 36, 911 - 921 (1957)
113. H.G. McCANN: Reactions of fluoride ion with hydroxyapatite. J. biol. Chem., 201, 247 - 259 (1953)
114. H.G. McCANN and F.A. Bullock: Reactions of fluoride ion with powdered enamel and dentine. J. dent. Res., 34, 59 - 67 (1955)
115. H.G. McCANN and F.A. Bullock: The effect of fluorine ingestion on the composition and solubility of mineralized tissues of the rat. J. dent. Res., 36, 391 - 398 (1957)
116. E.V. McCOLLUM, N. Simmonds, J.E. Becker and R.W. Bunting: The effect of additions of fluorine to the diet of the rat on the quality of the teeth. J. biol. Chem., 63, 553 - 562 (1925)
117. J. MacGREGOR and B.E.C. Nordin: Equilibration studies with human bone powder. II. The role of the carbonate ion. J. biol. Chem., 237, 2704 - 2708 (1962)
118. F.S. McKAY and G.V. Black: An investigation of mottled teeth: an endemic developmental imperfection of the enamel of the teeth heretofore unknown in the literature of dentistry. Dent. Cosmos, 58, 477 - 484, 627 - 644, 781 - 792, 894 - 904 (1916)
119. F.S. McKAY: The establishment of a definite relation between enamel that is defective in structure, as mottled enamel, and its liability to decay. II. Dent. Cosmos, 71, 747 - 744 (1929)

120. B.J. MacNULTY, G.F. Reynolds and E.A. Terry: Polarographic determination of fluoride. Nature, 169, 888 - 889 (1952)
121. B.J. MacNULTY, G.F. Reynolds and E.A. Terry: The polarographic determination of fluoride. Part I. Basic principles of the method: application to the cathode-ray polarograph. Analyst, 79, 190 - 198 (1954)
122. R.S. MANLY: Relation of refractive index to density in dental hard tissues. J. Amer. chem. Soc., 60, 2884 - 2886 (1938)
123. R.S. MANLY and H.C. Hodge: Density and refractive index studies of dental hard tissues. I. Methods for separation and determination of purity. J. dent. Res., 18, 203 - 211 (1939)
124. J.N. MANSBRIDGE: Sex differences in the prevalence of dental caries. Brit. dent. J., 106, 303 - 308 (1959)
125. S. Ye. MARKUS: Pogloshchenie ftoristogo natriia tverdymi zubnymi i kostnymi tkaniami. Stomatologiya, Mosk., 16, No.4, 51 - 55 (1937)
126. T.M. MARTHALER and R. Zilio: Enamel solubility studies in vivo: method error and effect of topically applied fluorides. Helv. odont. Acta, 4, 27 - 32 (1960)
127. S. MEGREGIAN: Fluorine. Colorimetric determination of non-metals, 231 - 259, ed. by D.F. Boltz. Interscience Publishers, New York, 1958
128. J. MIDDLETON: On fluorine in bones, its source, and its application to the determination of the geological age of fossil bones. Quart. J. geol. Soc., 1, 214 - 216 (1845)
129. G.W.C. MILNER: The principles and applications of polarography and other electroanalytical processes. Longmans Green and Co., London, 1957

130. R.F. MILTON: Titrimetric estimation of fluorine. Analyst, 74, 54 (1949)
131. C.L. MOROZZO: Analisi chimica del dente fossile fatta dal dottor Morrechini. Mem. mat. fis. soc. Ital. Sci., 10, 166 - 172 (1803)
132. R.K. MORTON: Alkaline phosphatase of milk. I. Association of the enzyme with a particulate lipoprotein complex. Biochem. J., 55, 786 - 795 (1953)
133. H.R. MÜHLEMAN and K.G. König: Caries symposium Zürich: the present status of caries prevention by fluoride-containing dentifrices. H. Huber, Berne, 1961
134. D.K. MYERS: Carboxyl ester cleavage (survey). The enzymes, 2nd edition ed. by P.D. Boyer, H. Lardy and K. Myrbäck, 4, 475 - 483. Academic Press, New York, 1960
135. H.M. MYERS, J.G. Hamilton and H. Becks: A tracer study of the transfer of F^{18} to teeth by topical application. J. dent. Res., 31, 743 - 750 (1952)
136. M.M. NACHLAS and A.M. Seligman: Evidence for the specificity of esterase and lipase by the use of three chromogenic substrates. J. biol. Chem., 181, 343 - 355 (1949)
137. Z. NAGY and E. Pólyik: A devaványai ivóvíz speciális vizsgálata fémnyomokra. Fogorv. Szemle, 48, 154 - 156 (1955)
138. W.F. NEUMAN, M.W. Neuman, E.R. Main, J. O'Leary and F.A. Smith: The surface chemistry of bone. II. Fluoride deposition. J. biol. Chem., 187, 655 - 661 (1950)
139. W.F. NEUMAN and M.W. Neuman: The chemical dynamics of bone mineral. University of Chicago Press, Chicago, 1958
140. W.F. NEUMAN, T.Y. Toribara and B.J. Mulryan: Synthetic hydroxyapatite crystals. I. Sodium and potassium fixation. Arch. Biochem. Biophys., 98, 384 - 390 (1962)

141. F.K. NIUSHKO and G.D. Ovrutskii: O vliianii na zuby vody s vysokim sodержaniem ftora. Stomatologiya, Mosk., 31, No.2, 24 - 25 (1952)
142. K.P. OAKLEY and C.R. Hoskins: New evidence on the antiquity of Piltdown man. Nature, 165, 379 - 382 (1950)
143. J. PAUL and P. Fottrell: Tissue-specific and species-specific esterases. Biochem. J., 78, 418 - 424 (1962)
144. A.S. POSNER and A. Perloff: Apatites deficient in divalent cations. J. Res. nat. Bur. Stand., 58, 279 - 286 (1957)
145. B.V. RAMACHANDRAN, L. Engström and G. Agren: Fractionation of $DF^{32}P$ -binding proteins of rat liver cell fractions by DEAE-cellulose chromatography. Biochem. Pharmacol., 12, 167 - 172 (1963)
146. W. RATHJE: Zur Verminderung der Kariesanfälligkeit durch fluorhaltiges Trinkwasser. Dtsch. zahnärztl. Z., 7, 693 - 701 (1952)
147. J. READ: Fluorine in the human skeleton. Nature, 172, 1156 (1953)
148. J.M. REINER, K.K. Tsuboi and P.B. Hudson: Acid phosphatases. IV. Fluoride inhibition of prostatic acid phosphatase. Arch. Biochem. Biophys., 56, 165 - 183 (1955)
149. L. RICHELLE and M.J. Dallemagne: Le calcium échangeable de la substance minérale de l'os étudiée à l'aide du ^{45}Ca . IV. Étude critique des conditions expérimentales. Bull. Soc. Chim. biol., 40, 1133 - 1151 (1958)
150. E. RÖNNHOLM: The amelogenesis of human teeth as revealed by electron microscopy. II. The development of the enamel crystallites. J. Ultrastructure Res., 6, 249 - 303 (1962).

151. H.M. ROOTARE, V. R. Dietz and F.G. Carpenter: Solubility product phenomena in hydroxyapatite-water systems. J. Coll. Sci., 17, 179 - 206 (1962)
152. G.H. ROVELSTAD, J.H. Geller and A.H. Cohen: Caries susceptibility tests, hyaluronidase activity of saliva and dental caries experience. J. dent. Res., 37, 306 - 311 (1958)
153. E.B. SANDELL and I.M. Kolthoff: Microdetermination of iodine by a catalytic method. Mikrochim. Acta, 1, 9 - 25 (1937)
154. A. SAVORY and F. Brudevold: The distribution of nitrogen in human enamel. J. dent. Res., 38, 436 - 442 (1959)
155. M. SCHLAMOWITZ: Enzymatic determination of fluoride. Pharmacology and toxicology of uranium compounds, ed. by C. Voegtlin and H.C. Hodge, 183 - 184. National nuclear energy series, VI, 1. McGraw-Hill, New York, 1949
156. F. SCHÖNHEYDER and K. Volqvartz: Studies on the lipolytic enzyme action. I. Kinetics of pancreatic lipase activity on an ester in homogeneous aqueous solution. Biochim. Biophys. Acta, 6, 147 - 165 (1950)
157. S. SCHWIMMER: Chronometric integrals of product-inhibited enzyme reactions and the hydrolysis of S-ethyl-L-cysteine. Biochim. Biophys. Acta, 48, 132 - 138 (1961)
158. D.B. SCOTT, R.G. Picard and R.W.G. Wyckoff: Studies of the action of sodium fluoride on human enamel by electron microscopy and electron diffraction. U.S. Publ. Hlth Rep., 65, 43 - 56 (1950)
159. S. SELLMAN: Effect of drying and heating on solubility of dental enamel. Odont. Revy, 4, 344 - 349 (1953)

160. J.H. SHAW, O.P. Gupta and M.E. Meyer: High fluoride content of teeth from communities with low fluoride water supplies. Amer. J. clin. Nutr., 4, 246 - 253 (1956)
161. C.E. SHOEMAKER: Polarographic determination of traces of fluoride and iron. Anal. Chem., 27, 552 - 556 (1955)
162. L. SINGER and W.D. Armstrong: Determination of fluoride: procedure based upon diffusion of hydrogen fluoride. Anal. Chem., 26, 904 - 906 (1954)
163. L. SINGER and W.D. Armstrong: Determination of fluoride in serum. Anal. Chem., 31, 105 - 109 (1959)
164. L. SINGER and W.D. Armstrong: Modified diffusion method for analysis of fluoride. J. dent. Res., 41, 910 (1962)
165. H.V. SMITH and W.B. Davey: The development and use of the bone filter for removing fluorine from drinking water. Univ. Ariz. Agric. Exp. Sta. Bull., No. 81, 247 - 292 (1939)
166. M.C. SMITH, E.M. Lentz and H.V. Smith: The cause of mottled enamel. Science, 74, 244 (1931)
167. Q.T. SMITH, W.D. Armstrong and L. Singer: Inhibition of salivary and prostatic acid phosphatase and yeast enolase by low fluoride concentrations. Proc. Soc. exp. Biol. Med., 102, 170 - 173 (1959)
168. R.F. SOGNAES: The organic elements of enamel. II. The organic framework of the internal part of the enamel with special regard to the organic basis for the so-called tufts and Schreger's bands. J. dent. Res., 28, 549 - 557 (1948)
169. R. SÖREMARK and N. Andersson: Uptake and release of vanadium from intact human enamel following $^{48}\text{V}_2\text{O}_5$ application in vitro. Acta odont. scand., 20, 81 - 93 (1962)

170. R.C. SPECHT: Interaction of fluoride ions and ground glass. Anal. Chem., 28, 1015 - 1017 (1956)
171. M.V. STACK: Organic constituents of enamel. J. Amer. dent. Ass., 48, 297 - 306 (1954)
172. H. STEGEMANN and G.F. Tung: Abtrennung von Fluorwasserstoff durch Diffusion und Mikroanalyse von Fluorid. Hoppe-Seyler's Z. physiol. Chem., 315, 222 - 227 (1959)
173. H. STETTER: Enzymatische Mikroanalyse des Fluor-ions. Chem. Ber., 81, 532 - 540 (1948)
174. G.K. STOOKEY and J.C. Muhler: Effect of molybdenum on fluoride retention in the rat. Proc. Soc. exp. Biol. Med., 109, 268 - 271 (1962)
175. G.K. STOOKEY, R.A. Roberts and J.C. Muhler: Synergistic effect of molybdenum and fluoride on dental caries in rat. Proc. Soc. exp. Biol. Med., 109, 702 - 705, (1962)
176. J. STRAUB and P. Adler: Fluoride saturation rate of human enamel. J. dent. Res., 28, 310 - 312 (1949)
177. I. SUNDVALL-HAGLAND, F. Brudevold, W.D. Armstrong, D.E. Gardner and F.A. Smith: A comparison of the increment of fluoride in enamel and the reduction in dental caries resulting from topical fluoride applications. Arch. oral Biol., 1, 74 - 79 (1959)
178. A. SYRRIST: A seven-year report on the effect of topical applications of sodium fluoride on dental caries. Odont. Revy., 7, 386 - 396 (1956)
179. A. TIETZ and S. Ochoa: Fluorokinase and pyruvic kinase. Arch. Biochem. Biophys., 78, 477 - 493 (1958)

180. E. UNDERHAY, S.J. Holt, H. Beaufay and C. de Duve: Intracellular localization of esterase in rat liver. J. biophys. biochem. Cytol., 2, 635 - 637 (1956)
181. J.F. VOLKER, H.C. Hodge, H.J. Wilson and S.N. Van Voorhis: The adsorption of fluorides by enamel, dentin, bone and hydroxyapatite as shown by the radioactive isotope. J. biol. Chem., 134, 543 - 548 (1940)
182. P. WALLACE-DURBIN: The metabolism of fluorine in the rat using F^{18} as a tracer. J. dent. Res., 33, 789 - 800 (1954)
183. H.-G. WALTER: A simple laboratory method for preliminary screening of organic fluorides as to their enamel solubility reducing effect. Helv. odont. Acta, 2, 40 - 43 (1958)
184. J.H. WEIKEL Jr., W.F. Neuman and I. Feldman: The surface chemistry of bone. VIII. On the mechanism of ionic exchange. J. Amer. chem. Soc., 76, 5202 - 5207 (1954)
185. H.W. WHARTON: Recoveries of fluoride by microdiffusion: effect of heat and diffusion time. Anal. Chem., 35, 406 - 408 (1963)
186. H.H. WILLARD and O.B. Winter: Volumetric method for determination of fluorine. Ind. Eng. Chem., Anal. Ed., 5, 7 - 10 (1933)
187. H.H. WILLARD and J.A. Dean: Polarographic determination of aluminium: use of an organic reagent. Anal. Chem., 22, 1264 - 1267 (1950)
188. N.V. WOOD: Specific surfaces of bone, apatite, enamel and dentine. Science, 105, 531 - 532 (1947)
189. S.H. YOON, F. Brudevold, D.E. Gardner and F.A. Smith: Distribution of fluoride in teeth from areas with different levels of fluoride in the water supply. J. dent. Res., 39, 845 - 856 (1960)

CHAPTER 9

APPENDIXES

List of Publications

- A. Prefluoridation dental examinations.
Account of sampling procedure: Ayr and
Kilmarnock. Health Bull., Edinb., 16,
16 - 21 (1958)
- B. Preparation of enamel powder in water.
J. dent. Res., 37, 748 (1958)
- C. Differentiation of enamel and dentine
particles. Stain Technol., 34, 43 - 46
(1959)
- D. The polarography of arsonic acids.
J. electroanal. Chem., 1, 303 - 313 (1960)
- E. A polarographic method for fluoride.
Talanta, 5, 61 - 63 (1960)
- F. Measurement of caries progress. Arch. oral
Biol., 6, 7 - 11 (1961)
- G. Ultramicro determination of phosphate
solubility of enamel surface.
Experientia, 18, 29 (1962)

PRE-FLUORIDATION DENTAL EXAMINATIONS

Account of Sampling Procedure: Ayr and Kilmarnock

The proposed introduction of fluorine to the water supplies of four demonstration areas in Great Britain entailed prior dental examinations of pre-school and school children, to obtain base-line data for subsequent annual inspections. Kilmarnock was chosen as one of these four areas, and the neighbouring Burgh of Ayr, with approximately the same population was selected as a "control". Preliminary discussions with all concerned decided the sampling procedure. The Statistician of the Ministry of Health, who is a member of the Fluoridation Steering Committee, was consulted to ensure uniformity with the other three demonstration areas in England and Wales. In planning the investigation, it was agreed that the sample should consist of at least the following children in each Burgh:—

KILMARNOCK.

	Boys	Girls
3 year olds	100	100
4 " "	55	55
5 " "	55	55
6 " "	55	55
7 " "	44	44
8 " "	44	44
9 " "	44	44
10 " "	44	44
11 " "	33	33
12 " "	33	33
13 " "	33	33
14 " "	33	33

There are roughly 3,000 pre-school children in Kilmarnock and rather more than 7,000 school children, including some, especially in the advanced classes, who come in from the surrounding county area.

The birth registers were consulted and lists of births of 3 and 4 year olds, i.e. all those on the register having been born between 1st June 1950 and 31st May 1952 were made. These lists were carefully examined and compared with the lists of children on the Health Visitors' cards. Children who were known to have died, or removed permanently from the district were excluded, and added to the lists were any children now thought to be permanently in the area, who had come into it before the age of 6 months. Where information was incomplete, the Health Visitor paid a special visit.

The lists as they now stood were again reviewed and there were 1,061 entries. As only about 200 children were needed, every fourth name according to date of birth was extracted and this gave some 265 entries. The homes of all the children included in these 265 items were visited by the Health Visitors and the guardian was asked if he or she was prepared to bring the child in question to one or other of the Child Welfare Clinics, so that his or her teeth could be examined by the Visiting Dental Surgeon. A short account of the reason for the visit was given, varying with the amount of knowledge of the fluoridation project already possessed by the guardian. Of the 265 entries, just over 200 evinced a desire to co-operate and stated that they would attend.

The Dental Surgeons carried out the examinations in June 1955 at three centres. Each child was given the alternative of two dates and in some cases, a third. Arrangements were made for 25 children to attend at each session. The number who actually attended varied from 16 to 25 and eventually, of the first 200 children chosen for examination, 166 were submitted for examination. Some 8 of these children proved refractory and

could not be examined, so the effective number was reduced to 158.

It then proved necessary to draw another sample and this time, starting from the second on the list, every eighth name was drawn. The Health Visitors visited the homes of the children as the names were submitted to them, and eventually from 58 homes visited, 34 children were presented, of which number 2 could not be examined as they proved non-co-operative. The reason for the small number is partly due to the fact that on at least two clinic sessions, it was raining incessantly. These were the last clinic sessions.

(As a matter of interest, some of the mothers brought their children the next week, although not requested, in the hope that the dental examination might still be made.)

The Dental Surgeons thus examined altogether 200 children from the sample drawn (this included 10 non-co-operators). It was considered desirable for them to examine, in addition, the children in the day nurseries, and 30 of these children were examined, i.e., all but 4 in the appropriate age group. (The latter 4 were off with infections).

With regard to the school children, each school was visited by a member of the clerical staff who, from the class registers, extracted every fifth name. The name in the first five was chosen at random. Thereafter, when moving from class to class, the sequence was maintained, i.e., if 2 remained in one class, the third in the succeeding class was the next chosen. In the extracts, any children with addresses outwith Kilmarnock and any children who had not lived all their lives in Kilmarnock, were excluded, and the next name following on the register was chosen. Altogether, 1,277 names were listed, and from this, the aim was to examine at least 880 chosen by random sample. (A few extra were placed on a supplementary list, so that the name of any absentee could be replaced by one from this list.)

The Dental Surgeons revisited each school towards the end of the Survey, to attempt to gather in the particulars of any absentees at the first visit who had returned to school. There was, unfortunately, an epidemic of mumps at this time and the result of this was that a considerable number of absentees were not seen at any of the visits.

The final figures were:—

Pre-School Children (including Nurseries)

	Chosen	Examined	Absent
Boys	148	108	40
Girls	145	112	33

School Children

	Chosen	Examined	Absent
Boys	458	444	14
Girls	461	446	15

AYR.

In general, the methods adopted in Ayr in the selection of the sample were similar to those used in Kilmarnock, but there were some differences in detail.

The pre-school sample required was:—

	Boys	Girls
1.6.51–31.5.52 (3 year old)	55	55
1.6.50–31.5.51 (4 year old)	55	55
1.6.49–31.5.50 (5 year old)	18	18 (see below)

The Health Visitors overhauled all the records of children in these groups and brought them up to date. Numbers in each case were considerably in excess of the sample required and reduction was made by the use of random numbers, after preliminary elimination of all who were not life-long residents and a very few who were considered as certain not to co-operate.

All children (approximately 60) attending the Nursery School (a few of these had already been selected as part of the sample) were dentally examined in addition to the random sample.

Each school was visited in turn (no special sequence), class registers obtained and arranged in order from the highest class to the lowest (e.g. Senior 3A, 3B, 3C—2A, 2B, 2C, etc. Primary 7A, B, 6A, B, etc.)

Registers were then scrutinised in order as above, and, disregarding all children born prior to 1st June, 1940, the name, class, address and date of birth of every fifth child was recorded.

All pupils at present resident outside the Burgh were then removed from the list. A further visit was then made to each school in order to eliminate from the remainder, those who had not been life-long residents in Ayr. Lists were then prepared according to age-groups.

The samples required in the school age-groups were:—

	Boys	Girls
1.6.49-31.5.50 (5 year old) ...	55 (37)	55 (37)
1.6.48-31.5.49 (6 " ") ...	55	55
1.6.47-31.5.48 (7 " ") ...	55	55
1.6.46-31.5.47 (8 " ") ...	44	44
1.6.45-31.5.46 (9 " ") ...	44	44
1.6.44-31.5.45 (10 " ") ...	44	44
1.6.43-31.5.44 (11 " ") ...	44	44
1.6.42-31.5.43 (12 " ") ...	33	33
1.6.41-31.5.42 (13 " ") ...	33	33
1.6.40-31.5.41 (14 " ") ...	33	33

Where the numbers were in excess, in any age-group, names were eliminated by the use of random numbers until the requisite sample remained. But unexpectedly in three instances, girls 1945-46, boys 1947-48, and girls 1949-50, the numbers, after elimination of non-residents, were insufficient. To correct this, a further visit was made to each school and the additional numbers required for the samples picked out by the use of random numbers, starting with the first name not already chosen.

Owing to the fact that most of the schools admit 5 year old entrants twice in the session, it was found that a considerable proportion of the 1949-50 group were not yet at school and a school sample was reduced to 37 boys and 37 girls and augmented by 18 boys and 18 girls not yet attending school.

Return visits were paid to schools to pick up absentees and, as far as practicable, pre-school children were offered an alternative appointment. Practically all the mothers of the pre-school children signified their intention of keeping their appointments but, as the following figures show, quite a considerable number failed to do so:—

Pre-School Children (including Nursery School)

	Chosen	Examined	Non-Attend-ers
Boys ...	143	108	35
Girls ...	145	109	36

School Children

Boys ...	424	423	1
Girls ...	419	413	6

General.

The parents of the school children were not informed specifically about the examination, as dental examination

in general in Scotland are carried out without previous notice to parents.

The whole procedure took place in the 6 weeks from mid-May to the end of June in both the Burgh of Kilmarnock and the control town of the Burgh of Ayr.

No particular difficulty was experienced but the visiting of the homes of pre-school children was time-consuming. It was felt that a reasonable cross-section of the dental condition of the children in the Burghs was obtained and that it will serve as useful base-line data for further investigations.

DENTAL EXAMINATIONS AND RESULTS.

Personnel: The dental examinations were carried out by Dr J. N. Mansbridge, Lecturer in Preventive and Social Dentistry in the Department of Social Medicine, and Mr C. P. Wallis, Lecturer in Preventive Dentistry in the Department of Dental Surgery of the University of Edinburgh.

Two assistants were also employed for clerical duties and the recording of examination data.

Equipment: The examinations were made using plane mirrors and No. 54 Ash probes. Since the examinations were carried out in schools and clinics, and the standards of accommodation varied considerably, each examiner carried a portable Angle-poise lamp with a 60 watt bulb. These were used throughout the Survey regardless of the standards of natural lighting available.

A portable dental chair and a portable headrest for attaching to an ordinary domestic chair completed the dental equipment.

Methods of Examination: The examinations were commenced in May and were completed by 1st July, 1955. The pre-school children were examined at Child Welfare Clinics, with the exception of a small number, who were examined at Nurseries in Kilmarnock, and a nursery school in Ayr.

The school children were examined at their respective schools.

The findings were dictated by the examiner to a clerical assistant who recorded these on a dental chart.

In assessing dental caries, no lesion was recorded as carious unless there was a definite breach of the enamel, together with palpable softening of the underlying dentine. In the case of pits and fissures, these were not recorded as carious unless some softness was palpable by probe, using light pressure. Hard surface defects of enamel were not recorded as being carious.

Using these criteria of dental caries, data were obtained for each tooth surface and each child examined, which indicated:—

- Each specific tooth surface decayed and/or filled.
- Each specific tooth that was present, non-carious, decayed or filled.
- Missing permanent teeth were assumed to have been carious, unless a history was obtained to indicate that tooth loss was due to other causes, such as, trauma or orthodontic extraction. The procedures adopted for the deciduous dentition are described on page 18.
- Unrupted teeth were recorded as such, but in the tabulated data which follow, all teeth which were unerupted or known to have been lost for reasons other than caries, have been excluded.

Results: The results of the dental survey of Kilmarnock and Ayr children are presented in terms of three indices.

- The total number of decayed, missing and filled teeth expressed as the rate *per 100 children*.
- The total number of decayed, missing and filled teeth expressed as the rate *per 100 teeth* examined.
- The percentage of children who were free from dental caries.

Pre-Fluoridation Dental Survey: 1955
Permanent Dentition

Table 1—Kilmarnock Boys

Age	Number of Children Examined	D.M.F. Teeth per 100 Children Examined	D.M.F. Teeth per 100 teeth Examined	Percentage of Children Caries-free
5	19*	16	4.84	94.7
6	56	61	11.89	69.6
7	57	128	14.72	38.6
8	49	200	17.28	18.4
9	42	274	19.93	11.9
10	47	368	21.33	4.2
11	49	406	19.01	6.1
12	30	537	21.27	3.3
13	33	633	22.92	15.1†
14	40	730	27.38	2.5

This high figure has been checked. It appears to be a chance occurrence, representing 5 children caries-free out of a total of 33 children.
A total of 47 five year old boys was examined, of whom 28 had *no* permanent teeth erupted.

Table 2—Kilmarnock Girls

Age	Number of Children Examined	D.M.F. Teeth per 100 Children Examined	D.M.F. Teeth per 100 teeth Examined	Percentage of Children Caries-free
5	34*	47	12.21	79.4
6	58	79	12.96	56.9
7	55	174	18.04	27.3
8	45	271	21.98	8.9
9	43	344	23.34	4.6
10	46	483	24.23	4.3
11	45	553	24.20	2.2
12	34	803	30.71	2.9
13	33	761	27.89	0.0
14	32	756	27.25	3.1

A total of 57 five year old girls was examined, of whom 23 had *no* permanent teeth erupted.

Table 3—Ayr Boys

Age	Number of Children Examined	D.M.F. Teeth per 100 Children Examined	D.M.F. Teeth per 100 teeth Examined	Percentage of Children Caries-free
5	17*	35	10.53	76.5
6	57	65	13.86	64.9
7	55	178	20.67	29.1
8	45	240	20.38	11.1
9	45	322	24.13	15.5
10	44	370	21.70	6.8
11	43	458	21.67	4.6
12	33	515	20.02	0.0
13	33	609	23.26	6.1
14	33	721	26.24	6.1

A total of 54 five year old boys was examined, of whom 37 had *no* permanent teeth erupted.

Table 4—Ayr Girls

Age	Number of Children Examined	D.M.F. Teeth per 100 Children Examined	D.M.F. Teeth per 100 teeth Examined	Percentage of Children Caries-free
5	17*	35	8.82	76.5
6	57	93	17.26	59.6
7	53	219	20.94	18.9
8	44	286	22.30	11.4
9	40	352	22.81	5.0
10	42	454	23.43	2.4
11	42	586	24.09	4.8
12	33	627	24.70	3.0
13	34	709	26.28	0.0
14	32	875	31.60	3.1

* A total of 57 five year old girls was examined, of whom 40 had *no* permanent teeth erupted.

The permanent and deciduous dentitions were tabulated separately to avoid the difficulties which arise as the result of exfoliation of the deciduous teeth after age of 5 years.

Deciduous Dentition: The data relating to the deciduous teeth have been presented for two distinct age-groups:—

(a) Those aged 3 and 4 years.

(b) Those aged 5 to 8 years (inclusive).

For the first age-group, the indices have been calculated, making the assumption that all missing teeth were extracted because of dental caries and the indices therefore relate to all 20 deciduous teeth.

In the 5 to 8 year age-group, the indices were calculated in respect of the deciduous first and second molars and canine caries.

The deciduous incisor teeth were excluded from all consideration in this age group since their absence may be due equally to natural exfoliation or extraction because of dental caries.

The indices therefore relate to twelve deciduous teeth only, and the assumption is made that missing molars or canine teeth in this age-group were extracted because of dental caries since natural exfoliation of these teeth does not normally occur within this age range.

The tabulated results for the deciduous dentition are presented in Tables 5 to 8.

DECIDUOUS DENTITION : 1955

Table 5—Kilmarnock Boys

Age	Number of Children Examined	D.M.F. Teeth per 100 Children Examined	D.M.F. Teeth per 100 teeth Examined	Percentage of Children Caries-free
<i>Pre-School Children</i>				
3	53	475	23.77	26.4
4	48	677	33.85	12.5
<i>School Children 5 to 8 years (inclusive) Deciduous Molars and Canine Teeth only</i>				
5	47	623	51.95	8.5
6	56	705	58.78	1.8
7	57	749	62.43	1.7
8	49	797	66.50	0.0

Table 6—Kilmarnock Girls

Age	Number of Children Examined	D.M.F. Teeth per 100 Children Examined	D.M.F. Teeth per 100 teeth Examined	Percentage of Children Caries-free
<i>Pre-School Children</i>				
3	52	350	17.50	32.7
4	57	651	32.54	14.0
<i>School Children 5 to 8 years (inclusive) Deciduous Molars and Canine Teeth only</i>				
5	57	668	55.70	5.3
6	58	729	60.77	5.2
7	55	724	60.30	1.8
8	45	827	68.89	0.0

Table 7—Ayr Boys

Age	Number of Children Examined	D.M.F. Teeth per 100 Children Examined	D.M.F. Teeth per 100 teeth Examined	Percentage of Children Caries-free
<i>Pre-School Children</i>				
3	41	454	22.41	31.7
4	47	698	34.89	10.6
<i>School Children 5 to 8 years (inclusive) Deciduous Molars and Canine Teeth only</i>				
5	54	613	51.08	7.4
6	57	761	63.45	1.7
7	55	753	62.73	3.6
8	45	771	64.26	0.0

Table 8—Ayr Girls

Age	Number of Children Examined	D.M.F. Teeth per 100 Children Examined	D.M.F. Teeth per 100 teeth Examined	Percentage of Children Caries-free
<i>Pre-School Children</i>				
3	34	415	20.73	32.3
4	53	617	30.85	9.4
<i>School Children 5 to 8 years (inclusive) Deciduous Molars and Canine Teeth only</i>				
5	57	612	51.02	3.5
6	57	693	57.75	5.3
7	53	774	64.46	0.0
8	44	830	69.13	2.3

Fluoridation of the water supplies started in April 1956—approximately 11 months after first base-line exam-

inations in May-June 1955. In May-June 1956, i.e., to 6 weeks after the start of fluoridation, the second annual examinations of the children began. As fluoridation has been in operation for so short a time, it was felt that the examinations could also be used as base-line data.

As far as possible, the children examined in 1955 were re-examined in 1956. In the case of the pre-school 3 year olds, in 1955, it was possible to examine almost all the few that were unavailable being replaced by others from last year's list of surplus names. The 4 year olds in the pre-school group were in 1956 examined as 5 year old school entrants. It was necessary to choose a completely new 3 year old group in 1956, and this was done in the same manner as in 1955.

With regard to the school children, it is to be understood that each group was a year older in 1956, the 5 year olds becoming the sixes, and so on. The 4 year olds last year became the 5 year olds this year, as has been explained, and the 14 year olds passed out of the survey at the end of the age range. It was only necessary to find replacements for 39 school children and these were chosen from the surplus list of the year before.

The personnel, equipment and methods of examination were the same as those employed for the original base-line (pre-fluoridation) survey.

The results of the 1956 survey are summarised in Table 9 to 16.

FLUORIDATION DENTAL SURVEY: 1956

Permanent Dentition

Table 9—Kilmarnock Boys

Age	Number of Children Examined	D.M.F. Teeth per 100 Children Examined	D.M.F. Teeth per 100 teeth Examined	Percentage of Children Caries-free
5	20*	35	12.50	80.0
6	42	38	7.37	73.8
7	63	191	20.76	25.4
8	57	258	22.01	12.3
9	47	300	21.59	4.3
10	43	402	23.22	7.0
11	49	488	22.83	0.0
12	48	619	24.61	2.1
13	33	815	29.89	0.0
14	36	822	29.66	2.8

* A total of 47 five year old boys was examined, of whom 27 had no permanent teeth erupted.

Table 10—Kilmarnock Girls

Age	Number of Children Examined	D.M.F. Teeth per 100 Children Examined	D.M.F. Teeth per 100 teeth Examined	Percentage of Children Caries-free
5	20*	35	11.86	80.0
6	57	116	15.98	49.1
7	56	202	20.18	25.0
8	59	290	23.30	13.6
9	45	393	25.11	2.2
10	46	478	25.37	6.5
11	44	675	27.97	2.3
12	47	860	32.85	0.0
13	35	1006	36.70	2.9
14	34	988	35.90	0.0

* A total of 48 five year old girls was examined, of whom 28 had no permanent teeth erupted.

Table 11—Ayr Boys

Age	Number of Children Examined	D.M.F. Teeth per 100 Children Examined	D.M.F. Teeth per 100 teeth Examined	Percentage of Children Caries-free
5	21*	24	8.20	80.9
6	54	76	14.29	63.0
7	54	207	23.48	22.2
8	52	273	23.87	15.4
9	43	363	25.79	9.3
10	46	404	24.31	8.7
11	44	491	23.10	2.3
12	42	674	26.88	4.8
13	31	755	27.96	0.0
14	32	809	29.50	0.0

* A total of 59 five year old boys was examined, of whom 38 had no permanent teeth erupted.

Table 12—Ayr Girls

Age	Number of Children Examined	D.M.F. Teeth per 100 Children Examined	D.M.F. Teeth per 100 teeth Examined	Percentage of Children Caries-free
5	23*	26	6.98	82.6
6	57	68	13.00	63.2
7	57	212	21.49	26.3
8	51	353	26.16	2.0
9	51	373	23.40	3.9
10	42	505	26.67	2.4
11	41	595	24.65	2.4
12	40	778	30.08	5.0
13	34	909	34.14	2.9
14	29	928	33.46	0.0

* A total of 57 five year old girls was examined, of whom 34 had no permanent teeth erupted.

Table 13—Kilmarnock Boys

Age	Number of Children Examined	D.M.F. Teeth per 100 Children Examined	D.M.F. Teeth per 100 teeth Examined	Percentage of Children Caries-free
<i>Pre-School Children</i>				
3	53	517	25.85	22.6
4	75	724	36.20	12.0
<i>School Children 5 to 8 years (inclusive) Deciduous Molar and Canine Teeth only</i>				
5	47	694	57.80	8.5
6	42	688	57.34	9.5
7	63	768	64.02	1.6
8	57	823	68.57	1.7

Table 14—Kilmarnock Girls

Age	Number of Children Examined	D.M.F. Teeth per 100 Children Examined	D.M.F. Teeth per 100 teeth Examined	Percentage of Children Caries-free
<i>Pre-School Children</i>				
3	59	388	19.41	44.1
4	59	614	30.68	11.9
<i>School Children 5 to 8 years (inclusive) Deciduous Molar and Canine Teeth only</i>				
5	48	627	52.36	6.2
6	57	756	63.01	3.5
7	56	805	67.11	1.7
8	59	842	70.20	1.7

Table 15—Ayr Boys

Age	Number of Children Examined	D.M.F. Teeth per 100 Children Examined	D.M.F. Teeth per 100 teeth Examined	Percentage of Children Caries-free
<i>Pre-School Children</i>				
3	67	515	25.75	28.4
4	59	756	37.80	10.2
<i>School Children 5 to 8 years (inclusive) Deciduous Molar and Canine Teeth only</i>				
5	59	639	53.25	5.1
6	54	722	60.19	1.8
7	54	843	70.22	1.8
8	52	787	65.54	1.9

Table 16—Ayr Girls

Age	Number of Children Examined	D.M.F. Teeth per 100 Children Examined	D.M.F. Teeth per 100 teeth Examined	Percentage of Children Caries-free
<i>Pre-School Children</i>				
3	53	521	26.04	22.6
4	49	602	30.10	12.2
<i>School Children 5 to 8 years (inclusive) Deciduous Molar and Canine Teeth only</i>				
5	57	656	54.68	3.5
6	57	732	60.96	0.0
7	57	753	62.72	3.5
8	51	871	72.55	0.0

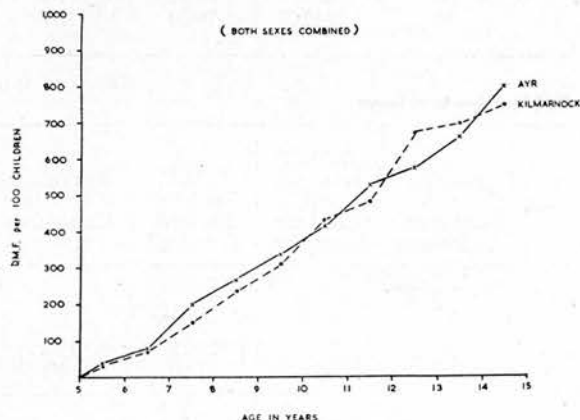
COMMENTS

Since fluoridation of the Kilmarnock Water Supply only began in April 1956, no observable effect upon the incidence of dental caries in Kilmarnock children could be expected by the time the second dental survey took place in May and June of the same year.

The Incidence of Dental Caries 1955: The data presented in Tables 1 to 8 reveal that there are no consistent differences in the incidence of dental caries of either the permanent or deciduous teeth between Kilmarnock and Ayr children. Such differences as do occur would appear to be entirely random.

A comparison of the dental caries incidence of the permanent teeth of Kilmarnock and Ayr children in 1955 is presented graphically in Figure 1 (for convenience both sexes have been combined.)

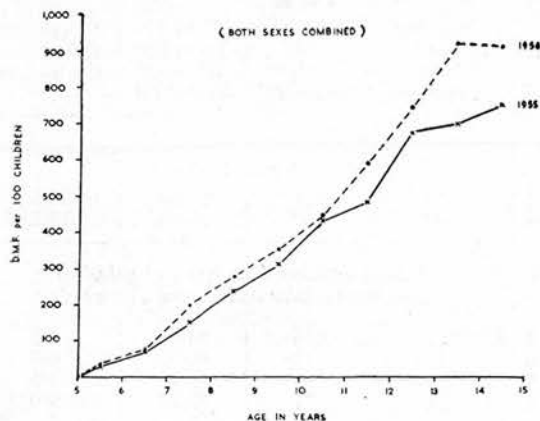
Figure 1
Incidence of Dental Caries, Ayr and Kilmarnock, 1955



The Incidence of Dental Caries 1956: The results of the 1956 Survey are presented in Tables 9 to 16. As in 1955, the findings in 1956 also show no consistent differences in the incidence of dental caries between Kilmarnock and Ayr children.

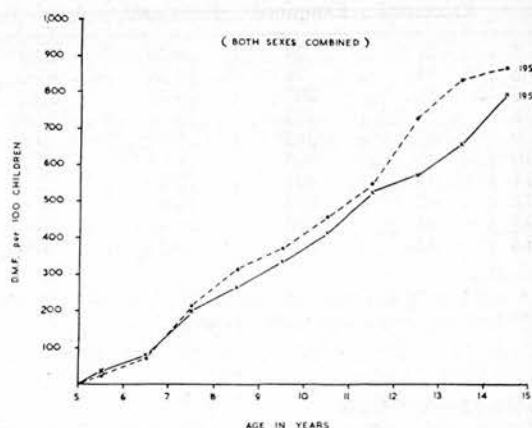
However, in both towns there is evident, a generally consistent increase in dental caries incidence of the permanent teeth in 1956, as compared with 1955. Figures II

Figure 2
Incidence of Dental Caries, Kilmarnock, 1955 and 1956



and III illustrate this increase in dental caries of the permanent teeth in both Kilmarnock and Ayr children.

Figure 3
Incidence of Dental Caries, Ayr, 1955 and 1956



The consistency of this increase at the various ages such as to suggest strongly that this increase in dental caries is not a chance effect but a real increase in incidence of this disease.

In the deciduous teeth however, the differences in dental caries in 1956, as compared with 1955 are less consistent, but nevertheless they suggest a trend towards a higher incidence in 1956 in both towns.

SUMMARY

Data are presented to show the incidence of dental caries by age and sex in Kilmarnock and Ayr children in the age range 3-14 years for 1955 and 1956.

Comparison of these data indicates that there are no important differences in dental caries experience between the Kilmarnock and Ayr samples in 1955 or 1956.

Furthermore, comparison of the data for 1956 with those for 1955 show in both Kilmarnock and Ayr, a consistent increase in incidence of dental caries of the permanent dentition, while a trend, suggestive of a similar increase is observed in the deciduous teeth.

The high incidence of dental caries in both Kilmarnock and Ayr is to be noted, where, by the age of 14 years nearly a third of the permanent teeth have already been attacked.

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J. N. MANSBRIDGE.
B. R. NISBET.
C. P. WALLIS.

Footnote.—We are indebted to Professor J. H. F. Brotherston of the Department of Public Health and Social Medicine and to Professor A. C. W. Hutchinson of the Department of Dental Surgery of the University of Edinburgh for their helpful co-operation and to the local authorities concerned for the facilities afforded.

BRITISH DIVISION OF THE
INTERNATIONAL ASSOCIATION
FOR DENTAL RESEARCH

PROCEEDINGS OF THE SIXTH ANNUAL
MEETING

APRIL 10TH AND 11TH, 1958

IVOR H. KRAMER, EDITOR, BRITISH
DIVISION

Sutherland Dental School, King's College
Newcastle-Upon-Tyne

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PROCEEDINGS OF THE SIXTH ANNUAL MEETING

APRIL 10TH AND 11TH, 1958

SUTHERLAND DENTAL SCHOOL, KING'S COLLEGE, NEWCASTLE-UPON-TYNE

Compiled by Ivor H. Kramer, Editor, British Division

1. PREPARATION OF ENAMEL POWDER IN WATER.—*C. P. Wallis, Department of Biochemistry and School of Dental Surgery, University of Edinburgh.* The Manly and Hodge separation (*J. D. Res.* 18: 133, 1939) has been modified to avoid drying the enamel. The crowns of extracted teeth are cleaned and ground under water in an electric mortar and pestle at 1° C., pouring off the suspension at 15-minute intervals. The powder is transferred to a 50 ml. centrifuge tube, washed with acetone, and centrifuged at 4,000 g for 15 minutes at 5° C. in bromoform/acetone of density 2.7. The dentine can be poured off with the liquid and each fraction is again centrifuged in the bromoform mixture. The enamel sediments are washed with acetone and water and stored at 0° C.

2. REMOVAL OF FLUORIDE FROM SOLUTIONS OF SODIUM AND STANNOUS FLUORIDE BY POWDERED ENAMEL.—*S. A. Leach, Turner Dental School, Manchester, F. A. Smith, D. E. Gardner, and H. C. Hodge, University of Rochester, School of Medicine and Dentistry, Rochester, N. Y.* Hatton, Nebergall, and Muhler (*J. D. Res.* 34: 350, 1955) reported that powdered enamel removed more fluoride from solutions of stannous fluoride than from solutions of sodium fluoride which initially contained stoichiometrically equivalent amounts of fluoride. Shaking powdered enamel in the fluoride solutions increased the pH, markedly for stannous fluoride and detectably for sodium fluoride. On increasing the pH of stannous fluoride solutions in the absence of enamel, precipitates were formed which contained sufficient fluoride that might account, at least in part, for the greater amount of fluoride removed from stannous fluoride solutions. The fluoride removed as precipitate from a series of stannous fluoride solutions on increasing the pH was measured and the fluoride removed at any arbitrarily chosen series of initial fluoride concentrations could be found by interpolation. From the data of Hatton and co-workers, in stannous fluoride solutions shaken with enamel, the fluoride similarly removed could be found at the same interpolated initial concentrations. No discrimination could be drawn between data from stannous and sodium fluoride solutions at the lower concentrations of fluoride. At the higher concentrations more fluoride may have been taken up from the stannous fluoride solutions; however, the differences are not great and the higher initial acidity may have been responsible.

DIFFERENTIATION OF ENAMEL AND DENTINE PARTICLES

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Received for publication August 15, 1958

ABSTRACT.—A method, based on the periodic acid-leucofuchsin reaction, is described, by which dentine particles may be selectively stained with neutral solutions. The method is suitable for pulverized teeth, containing particles with dimensions greater than $1-2\mu$. It can be applied also to sections. The dentine is stained red by treatment with a buffered neutral solution of periodic acid followed by a dilute solution of unreduced fuchsin; the excess fuchsin being removed by washing with water. The enamel is either unstained or stained a light pink. The loss of material caused by the usual acidic solutions is reduced to negligible proportions.

INTRODUCTION

During the investigation of methods for separating the enamel and dentine of pulverized teeth, it became necessary to have a simple method for assessing the purity of the preparations. Manly and Hodge (1939) described the use for this purpose of a liquid with a refractive index between that of enamel and dentine; they estimated the degree of contamination by counting particles of each type.

In the present work, the particles were considerably smaller and this method was found to be difficult and tedious to apply. Attention was therefore turned to the method described by Hutton (1953) which made use of the periodic acid-Schiff reaction. These reagents are strongly acid and dissolve both enamel and dentine particles.

It was assumed at first that etching by acid or other means would be essential for the reaction to occur, and selective staining of the dentine was attempted using partial extraction with ethylene diamine and neutralized reagents. However, the results were better when the extraction was omitted, showing that periodate is capable of attacking intact dentine. The method finally developed is simple and rapid and the exact conditions for its use do not appear to be critical.

PROCEDURE

Reagents

1. 0.5% periodic acid adjusted to pH 7 with 0.1 N ammonium acetate buffer.
2. 0.02% basic fuchsin in water.

Material

The entire root and pulp were removed under running tap water from all teeth shortly after extraction. The crowns were stored and ground under water, and the powder was not dried at any stage prior to staining (except in the re-

covery experiments). Some samples of powder were centrifuged in bromoform (specific gravity 2.86) in order to obtain pure enamel powder for control experiments. All preparations of tooth powder were used within 1 wk of extraction of the teeth.

Method

About 20 mg of powder was incubated for 5 min at 50°C with 4 ml of the periodate solution in a centrifuge tube. The powder was centrifuged down, washed twice with water and incubated for a further 20 min at 50°C with 4 ml of fuchsin solution. After washing a further 5 times with water, the powder was spread on a slide, allowed to dry, and mounted in Canada balsam.

RESULTS

Photomicrographs of stained tooth powder are shown in Fig. 1. Dentine stains a bright red in the larger particles, and rather paler in the smaller particles. Enamel shows a faint pink in bulk, and in the largest particles under the microscope. The differentiation of enamel is improved by the mounting medium, in which enamel particles appear more refractile than dentine particles.

As a check, the method was applied to a ground section of a tooth which had been fixed in formalin. The dentine was heavily stained, and the enamel was unstained apart from a few small areas which were pink.

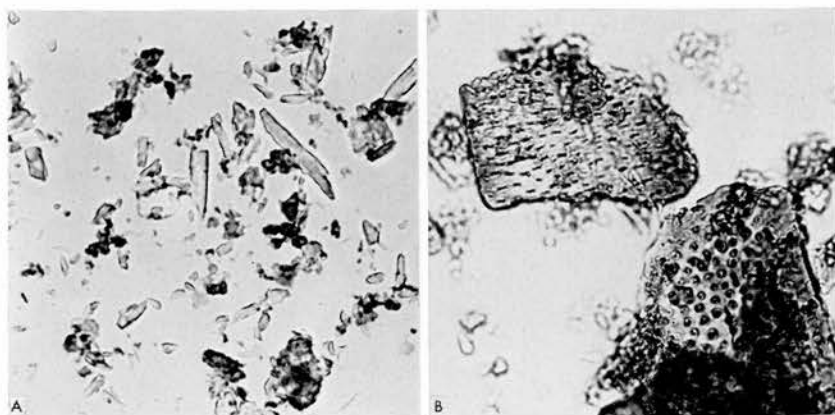


FIG. 1. Stained tooth powder, $\times 300$. (a) Mixed enamel and dentine particles; dentine particles are dark: (b) Large dentine particles. For photographic purposes, these samples were stained with 0.05% fuchsin.

TABLE 1. LOSS IN WEIGHT DURING STAINING OF TOOTH POWDER

Sample no.	1	2	3
Initial weight, mg	14.3	24.3	41.2
Loss, mg	0.4	0.9	1.3
Loss, %	2.8	3.7	3.2

The solubility of the powder in the solutions used was investigated by measuring the loss in weight during staining. Samples of tooth powder were washed in acetone, dried at 60°C and weighed. The powder was then suspended in water, stained as described above, washed in acetone and dried again at 60°C. The results are shown in table 1.

DISCUSSION

The powder used in these experiments had a mean particle diameter in the range 10–20 μ , and difficulty had been experienced in distinguishing between enamel and dentine particles, the smaller particles being particularly troublesome in this respect. The method described was developed primarily to show the particles of dentine, which are usually smaller than the enamel particles in purified preparations of the latter. However there is also sufficient contrast between the two types of particles after staining to enable the purity of dentine preparations to be checked.

One of the aims of this investigation was to eliminate the dissolution of powder by acid reagents: the results in table 1 show that the amount of powder dissolved is negligible. The solubility of enamel in water is low, but the loss is considerably smaller than the amount which could be dissolved at equilibrium in the total volume of water used.

It is probable that the stain is confined to those areas in the teeth which contain appreciable amounts of organic matter, and that the staining in fact demonstrates the distribution of organic matter. There is also little doubt that the density of an enamel particle is primarily determined by its organic content. One may therefore expect that enamel particles which float in bromoform with a density lower than the density of bulk enamel will also stain more intensively. Fortunately it does not seem likely that the intensity of staining will approach that of dentine.

These considerations are of interest in developing a quantitative measurement of enamel purity. If stained enamel particles were dissolved in acid, it might be expected that the light absorption could be used to give a measure of the purity. This estimate will vary to some extent with the method of preparation of the enamel for the reasons already mentioned, but comparative estimates should be possible. Preliminary experiments on these lines have been encouraging.

ACKNOWLEDGEMENTS

The author is indebted to Prof. G. F. Marrian for the provision of laboratory facilities and to Mr. A. Turner for supplying fresh teeth.

REFERENCES

- HUTTON, W. E. 1953. A test for the purity of powdered enamel. *J. Dent. Res.*, **32**, 626–7.
MANLY, R. S., AND HODGE, H. C. 1939. Density and refractive index studies of dental hard tissues. I. Methods for separation and determination of purity. *J. Dent. Res.*, **18**, 133–41.

THE POLAROGRAPHY OF ARSONIC ACIDS

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The polarography of the arsonate group was first investigated by BREYER¹ who found reduction to occur irreversibly at potentials more negative than -1.0 V. The wave is unsatisfactory for measurement and later work has concentrated on substituted derivatives. KOLTHOFF² used *m*-nitrophenylarsonic acid to titrate thorium amperometrically, the decrease in height of the NO_2 wave being produced by precipitation of the thorium arsonate. More recently, MARUYAMA AND FURUYA³ described the polarographic behaviour of a series of substituted diphenylarsinic acids. It is of interest that the *o*-nitro derivatives showed distinct differences from the other isomers.

During a survey of the reactions of various arsonic acids with thorium it was observed that certain compounds exhibited an unexpected polarographic wave in acid solutions. The present work is a preliminary investigation of the nature of the waves shown by *o*-nitrobenzenearsonic acid and Thoron Reagent.

METHODS

The measurements were made with a Southern Instruments Ltd. cathode ray polarograph using an Ag/AgCl anode, and with a manual polarograph connected to the same cell; electrocapillary curves were determined with the latter by timing 50 drops. All solutions contained 0.02 *N* chloride and the cell temperature was maintained at 25° . The arsonic acids were used as purchased, recrystallisation having no apparent effect on the polarographic behaviour. Chloracetic acid was purified by fractional crystallisation.

RESULTS

o-Nitrobenzenearsonic acid

The cathode ray polarogram shows three peaks before the arsonate wave (Fig. 1). The first peak, at -0.22 V is due to the reduction of the nitro group. The second peak at -0.47 V is rather small; the peak at -0.75 V is double and its origin is not immediately apparent. In the manual polarogram (Fig. 2) the same three waves are seen, the third wave having a maximum. The start of the reduction of the arsonate group of *o*-arsanilic acid is also shown in Fig. 2. There was no evidence of current inhibition of the type described by SCHMID AND REILLEY⁴ although the increase of current during each drop was rather erratic in the region of the third wave.

An examination of the cathode ray polarograms of *m*-nitrobenzenearsonic acid,

o-arsanilic acid and benzenearsonic acid showed no peaks in the region of the second and third waves of *o*-nitrobenzenearsonic acid.

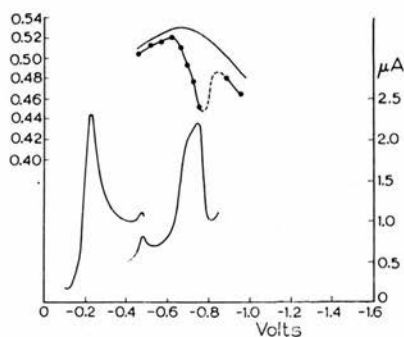


Fig. 1. Lower part: cathode ray polarogram of 0.001% *o*-nitrobenzenearsonic acid in 0.1 *M* chloracetate buffer half neutralised. Upper part: electrocapillary curves; ● 0.01% *o*-nitrobenzenearsonic acid in same buffer; continuous line, buffer only. Left ordinate is point reached on screen during sweep at which drop falls; 0.01 units = 40 msec approximately.

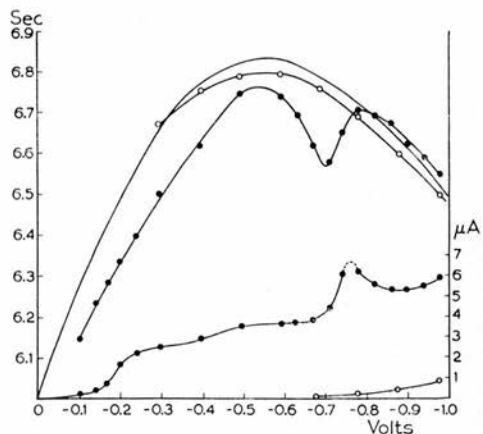


Fig. 2. Lower part: manual polarograms. Upper part: electrocapillary curves (left ordinate indicates drop-time); continuous line, 0.1 *M* chloracetate buffer half neutralised; ● 0.005% *o*-nitrobenzenearsonic acid in same buffer; ○ 0.005% *o*-arsanilic acid in same buffer.

In the presence of a surface active material (0.01% Lissapol N) the second and third waves in both types of polarogram were suppressed. A similar result was produced by the addition of an equivalent amount of a thorium salt. In a cationic buffer (0.1 *M* pyrazole) the height of these waves was reduced. Table I shows that the third peak disappears on dilution.

TABLE I

EFFECT OF THE CONCENTRATION OF *o*-NITROBENZENEARSONIC ACID ON THE HEIGHTS OF THE PEAKS IN THE CATHODE RAY POLAROGRAPH

Concentration $\mu\text{moles/l}$	Ratio of heights of peaks third/first
200	1.03
20	0.46
2	0.15

The effect of varying the height of the mercury column is shown in Table II. The change in height of the first and second waves is, within the experimental error, proportional to the square root of the head of mercury. The third wave (*i.e.* the diffusion current following the maximum) shows essentially no alteration: this is usually considered to be the criterion of a kinetic current.

It was apparent from the effects of surface active materials that some adsorption was occurring and an electrocapillary curve was therefore obtained (Fig. 2). This shows two maxima, and a minimum corresponding to the foot of the third wave.

TABLE II

EFFECT OF THE HEAD OF Hg ON THE MANUAL POLAROGRAPHIC WAVE HEIGHTS OF *o*-NITROBENZENEARSONIC ACID

Hg pressure cm	1st wave (-0.3 V) μA	2nd wave (-0.5 V) μA	3rd wave (-0.9 V) μA
24.6	1.95	0.45	1.65
32.9	2.15	0.65	1.85
41.6	2.3	0.8	1.85
51.6	2.55	0.85	1.8
57.0	2.6	0.95	1.8
64.6	2.8	0.95	1.8
69.4	2.85	1.0	1.8

The corresponding curve for *o*-arsanilic acid indicates some slight depression of the negative branch, suggesting adsorption of a positive ion.

In order to determine if the saw-tooth potential of the cathode ray polarograph affected the electrocapillary curve, the point on the screen at which the drop fell off was plotted against the corresponding potential (Fig. 1). The error of measurement is greater, and the curve is displaced by about 0.1 V, but the general shape is similar.

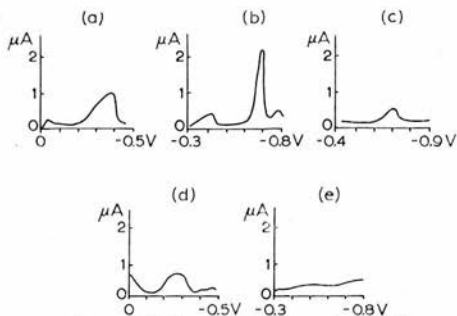


Fig. 3. Cathode ray polarogram of 0.001% Thoron Reagent in 0.1 N acetate buffer half neutralised. 0.02% Lissapol N added to (d) and (e).

Thoron Reagent: o-(2-hydroxy-3,6-disulpho-1-naphthylazo)-benzenearsonic acid

The cathode ray polarogram of Thoron Reagent shows three peaks in acid solution (Fig. 3). The first is double and has a small prewave (at -0.15 V), but a single well defined peak appears in its place if the pH of the solution is progressively raised, showing that this peak is due to reduction of the azo group. The second peak shows the unusual property of disappearing completely when the starting potential of the sweep is 0.15 V positive to the foot of the peak. The diminution of the second peak as the starting potential is advanced is closely correlated with the position of the start potential in relation to the second part of the first peak. Experience has shown that other peaks formed in the cathode ray polarograph are only decreased in height when the starting potential has passed the foot of the peak: the normal behaviour may be seen in Fig. 5. The third peak of Thoron Reagent is only partially visible on the screen of the standard cathode ray polarograph and has not been investigated; it appears to behave similarly to the second peak, however.

The effect of Lissapol N is to eliminate the second wave, and to decrease the second

part of the first wave. When sufficient detergent is added to eliminate the second part almost completely (Fig. 3(d) and (e)), the first part is depressed. Thorium has no effect on the peaks, provided precipitation does not occur.

The manual polarogram is shown in Fig. 4. The slope of the first wave indicates that it is irreversible (or possibly an unresolved double wave); the foot, centre and top correspond respectively to the prewave and the first and second parts of the first cathode ray peak. There is also a wave corresponding to the third peak: the second peak occurs at the foot of this wave.

The electrocapillary curve in Fig. 4 shows a distinct minimum in the region of the first wave; at potentials above and below this, there is evidence of adsorption.

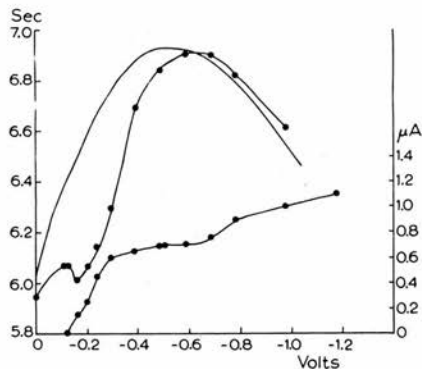


Fig. 4. Lower part: manual polarogram of 0.005% Thoron Reagent in 0.1 *N* acetate buffer half neutralised. Upper part: ● corresponding electrocapillary curve; continuous line, buffer only. Left ordinate indicates drop-time.

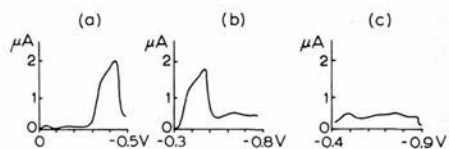


Fig. 5. Cathode ray polarogram of 0.001% ADNS in 0.1 *N* acetate buffer half neutralised

o-(1,8-dihydroxy-3,6-disulpho-2-naphthylazo)-benzenearsonic acid: ADNS

This compound, closely related to Thoron Reagent, shows in the cathode ray polarogram (Fig. 5) a first peak of similar shape, but there is no evidence of any peak

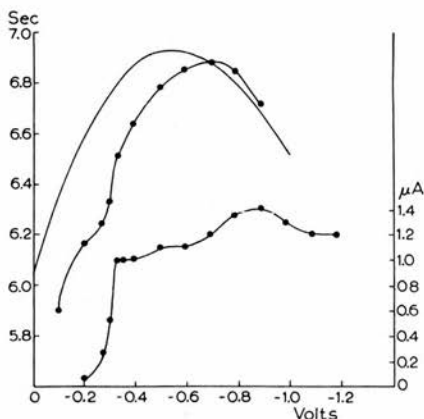


Fig. 6. Lower part: manual polarogram of 0.005% ADNS in 0.1 *N* acetate buffer half neutralised. Upper part: ● corresponding electrocapillary curve; continuous line, buffer only. Left ordinate indicates drop-time.

nalogous to the second peak of Thoron Reagent. The peak became rounded on addition of Lissapol N. The first wave of the manual polarogram (Fig. 6) has a rounded foot and a very sharply angled top. The electrocapillary curve (Fig. 6) has a small inflexion, but the extent of the adsorption at potentials above and below the first wave appears to be similar.

p-Dimethylaminoazobenzenearsonic acid showed a single well-defined wave in cetate buffer and was not investigated further.

DISCUSSION

The polarographic peaks which have been described are characterised by (1) pH dependence of the peak potential and (2) complete suppression in the presence of a surface active material. The first implies that a reduction process is involved, and the second that it is limited to adsorbed material. A catalytic hydrogen wave might satisfy these criteria, but is unlikely to occur at such potentials.

The manual polarogram of *o*-nitrobenzenearsonic acid presents a limiting adsorption current (Table II) which is less than the diffusion current, although there is an indication that the adsorption current may become diffusion limited at long drop times. The electrocapillary curve (Fig. 2) is similar to that of thallium⁵ and shows strong adsorption of a positively charged species before the reduction, but no adsorption after the reduction, which implies that the limiting reaction must precede the reduction. The limiting process is probably the rate of adsorption because (a) the limiting current is suppressed by the addition of a surface active material, and (b) the cathode ray peak (which will be proportional to the extent of adsorption) is relatively large compared with the limiting current. Presumably the adsorption occurs through the hydroxylammonium group, as the positively charged group, and also the arsonate group, which may account for the slowness of the reaction. Whatever the mechanism, however, it is clear that the adsorbed material is reduced more easily than the unadsorbed material.

When the limiting adsorption current is greater than the diffusion current, due to stronger adsorption, the conventional polarographic wave will follow the lower part of the adsorption step up to the diffusion current level and will then be cut off sharply. This is shown in the polarogram of ADNS (Fig. 6), and can occur without any difference in the half-wave potentials of the adsorbed and bulk material.

The peak given by Thoron Reagent appears to be due to the adsorbed layer being reduced less easily than material in the bulk solution. This has no effect on the shape of the manual polarogram (although the diffusion current is probably decreased by adsorption of the reduced form), or on the cathode ray polarogram which starts after the first wave, for in both cases no Thoron Reagent is available at the electrode surface for adsorption. However, when the sweep starts before the first wave, the adsorbed Thoron Reagent is reduced only at a more negative potential giving a peak without a diffusion current. The size of this peak (assuming there is no spike maximum) corresponds to a surface coverage of 100–200 sq. Å/molecule.

The results illustrate three distinct ways in which adsorption of a depolariser may affect a polarogram: the adsorbed compound may be more difficult to reduce, or more easily reduced, or there may be little difference in the reducibility. These possibilities are represented by Thoron Reagent, *o*-nitrobenzenearsonic acid and ADNS respectively.

Related phenomena were examined by BRDIČKA⁶, who derived an equation for the polarogram of a fully reversible reduction on the assumption that

$$i_a - i = I + A$$

where i_a is the diffusion current, i is the current at any specified potential, I is the contribution due to diffusion according to the ILKOVIČ equation and A is the contribution due to the adsorbed material. This equation implies that an increase in A will decrease the polarographic current i , so that reduction of the adsorbed material can hardly make a significant contribution to A , as assumed by BRDIČKA.

A similar relation, and a similar final equation is obtained if the assumption is made that adsorption decreases i in proportion to the amount of adsorbed material. The adsorption wave following the normal wave is then to be considered as the increase in i due to the removal of adsorbed depolariser.

It follows that adsorption of the reduced form should cause a decrease in the more negative part of the wave, resulting in an adsorption step at the start of the wave as seen in the cathode ray polarogram of Thoron Reagent. Methylene Blue gives a similar polarogram, and it appears possible that the double peak is a characteristic of strongly adsorbed compounds, whether in the oxidised or reduced form.

ACKNOWLEDGEMENTS

The author is indebted to Prof. G. F. MARRIAN, F.R.S., for the provision of laboratory facilities and Dr. J. E. B. RANGLES for advice. The work was assisted by a grant from the Scottish Hospitals Endowment Research Trust.

SUMMARY

The polarograms of certain *ortho*-substituted arsonic acids show unusual features which are attributed to adsorption on the basis of the corresponding electrocapillary curves. It is concluded that the adsorption of these compounds alters their reducibility and at the same time decreases the diffusion current; the three types of effect to be expected are illustrated by the arsonic acids investigated. Although desorption was observed the effect on the polarogram was minor.

RÉSUMÉ

Les polarogrammes de certains acides arsoniques substitués en *ortho* montrent des caractères extraordinaires que les auteurs, en se basant sur les courbes électrocapillaires correspondantes, attribuent à l'absorption. Ils concluent que l'absorption de ces composés change leur réductibilité et en même temps diminue le courant de diffusion. Les trois types d'effets auxquels on peut s'attendre sont illustrés par les acides arsoniques étudiés. Bien qu'une désorption fût observée, son effet sur le polarogramme était peu important.

ZUSAMMENFASSUNG

Die Polarogramme gewisser *ortho*-substituierter Arsonsäuren zeigen ungewöhnliche Eigenschaften, die, auf Grund der entsprechenden Elektrokapillarkurven, auf Adsorption zurückgeführt werden. Die Verfasser schliessen, dass die Adsorption dieser Verbindungen deren Reduzierbarkeit ändert und zugleich den Diffusionsstrom

vermindert; die drei zu erwartenden Auswirkungsarten werden durch die untersuchten Arsonsäuren beleuchtet. Obwohl Desorption beobachtet wurde war ihre Auswirkung auf das Polarogram unbedeutend.

REFERENCES

- ¹ B. BREYER, *Ber. deut. chem. Ges.*, 71 (1938) 163.
- ² I. M. KOLTHOFF AND R. A. JOHNSON, *J. Electrochem. Soc.*, 98 (1951) 138.
- ³ M. MARUYAMA AND T. FURUYA, *Bull. Chem. Soc. Japan*, 30 (1957) 650.
- ⁴ R. W. SCHMID AND C. N. REILLEY, *J. Am. Chem. Soc.*, 80 (1958) 2087.
- ⁵ A. N. FRUMKIN AND N. S. POLYANOVSKAYA, *Zhur. Fiz. Khim.*, 32 (1958) 157.
- ⁶ R. BRDIČKA, *Collection Czechoslov. Chem. Commun.*, 12 (1947) 522.

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A polarographic method for fluoride

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SEVERAL methods for the polarographic determination of fluoride have been proposed; many of these require relatively large amounts of fluoride. For microgram amounts Langer¹ described the first amperometric method, and Shoemaker² was able to measure 1–200 μg fluoride with a rotating platinum electrode. McNulty *et al.*³ described the determination of 0.1–40 μg of fluoride. None of these methods makes use of the full potentialities of the polarographs now available.

The basic reaction in all the most widely-used fluoride analyses is the complexing of a metal by fluoride, and the amount of uncomplexed metal is measured, in equilibrium methods (which are more sensitive than titrations), either directly or by adding an indicator. The effect of fluoride is thus to decrease the reading, and the estimated value is the difference between two large values, which reduces the accuracy of the measurement. The components of the system which increase on addition of fluoride are the fluoride complex and the free indicator. Since there are no known fluoride complexes which give a polarographic wave distinct from that of the metal, a survey has been made of polarographically reducible compounds capable of binding thorium, which forms one of the strongest fluoride-metal complexes.

Two substances have so far been found which, under appropriate conditions, give a polarographic wave increasing in height with added fluoride (Table I).

TABLE I.—OPTIMUM CHANGE IN POLAROGRAPHIC WAVE HEIGHT DUE TO FLUORINE

System	Change, $\mu\text{amp}/\mu\text{g F}$
Al + Solochrome Violet RS ³	–0.15
Th + Chrome Azurol S	+0.012
Th + <i>o</i> -nitrobenzenearsonic acid	+0.56

It is apparent that Chrome Azurol S is unsatisfactory, but it has been possible to develop a simple method for the determination of microgram amounts of fluoride with *o*-nitrobenzenearsonic acid.

The reagent is made up to contain:

- 0.006% *o*-nitrobenzenearsonic acid
- 0.00025M thorium nitrate
- 8 mg/litre fluoride as sodium fluoride
- 0.3N chloroacetic acid
- 0.15N sodium hydroxide
- 0.05N ammonium chloride (for Ag/AgCl anode)

The solution is stable for two weeks in polythene. Two ml of the sample is neutralised, if necessary, with perchloric acid using 1 drop of 0.002% paramethyl red as indicator, and 1 ml of reagent is added. The total volume should not exceed 3.4 ml. The peak heights are measured with a cathode-ray polarograph after de-oxygenation.

The polarogram of *o*-nitrobenzenearsonic acid has three peaks: the second is small and the third is double. The calibration curve for fluoride is derived from the ratio of the height of the second part of the third peak to the height of the first peak, and is essentially linear for 2–10 μg of fluoride.

One reagent solution was used for 5 determinations on pure fluoride solutions over a period of a fortnight: the results are shown in Table II.

TABLE II.—MEANS AND STANDARD DEVIATIONS OF THE RATIO OF PEAK HEIGHTS OF THE REAGENT AFTER ADDITION OF FLUORIDE

F added, μg	Peak ratio	Corrected for blank
Nil	0.161 \pm 0.002	—
1	0.192 \pm 0.005	0.031 \pm 0.003
10	0.578 \pm 0.006	0.417 \pm 0.006

It is apparent that the method is simple and accurate, although it has not been possible to take advantage of the full sensitivity of the polarograph. The procedure has been developed for use in conjunction with the diffusion separation of fluoride,⁴ which eliminates the relatively high blank values of the standard distillation separation.

The addition of fluoride to the reagent is necessary to avoid a flat portion of the calibration curve; without this addition the reagent could be used for 10–18 μg of fluoride.

Summary—A polarographic method is proposed for the determination of fluoride, based on the action of fluoride on a complex formed between thorium and *o*-nitrobenzenearsonic acid. Increasing amounts of fluoride liberate increasing amounts of the organic compound, of which the polarographic wave is utilised. The wave-height therefore increases with increasing amounts of fluoride, in contrast to existing polarographic methods for the ion.

Zusammenfassung—Eine polarographische Methode für Bestimmung von Fluorid wird vorgeschlagen. Das Verfahren beruht auf der Reaktion von Fluorid mit dem Thoriumkomplex von *o*-Nitrobenzolarsonsäure. Steigende Mengen Fluorid setzen steigende Mengen der organischen Verbindung in Freiheit. Die durch die organische Komponente verursachte polarographische Welle ist in ihrer Höhe proportional der Fluorid-Menge; dies ist gegenteilig zu allen bisherigen polarographischen Fluoridbestimmungen.

Resumé—Une méthode polarographique est proposée pour le dosage des fluorures; cette méthode est basée sur l'action du fluorure sur un complexe thorium-acide *o*-nitrobenzène arsonique. Des quantités croissantes de fluorure libèrent des quantités croissantes du composé organique dont on suit la vague polarographique. La hauteur de la vague augmente donc avec des quantités croissantes de fluorure, à l'opposé des méthodes polarographiques existant déjà pour cet ion.

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REFERENCES

- ¹ A. Langer, *Ind. Eng. Chem. Anal.*, 1940, **12**, 511.
- ² C. E. Shoemaker, *Analyt. Chem.*, 1955, **27**, 552.
- ³ B. J. MacNulty, G. F. Reynolds and E. A. Terry, *Analyst*, 1954, **79**, 190.
- ⁴ L. Singer and W. D. Armstrong, *Analyt. Chem.*, 1954, **26**, 904.

MEASUREMENT OF CARIES PROGRESS

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Abstract—The development of the carious process has been observed to occur with considerable regularity in large groups, but the irregular appearance of cavities in the individual has led to a general impression that caries occurs by chance. An attempt has been made to apply the group regularities to the individual subject: it is assumed only that caries is a continuous process. Using this assumption a measure of the aetiological agency can be derived which is found to be proportional under certain limited conditions to the DMF. It appears that this explains the fact that the DMF, although based on the past caries experience is also related to current caries incidence. The aetiological factors are divided into three groups related to the saliva (*a*), the tooth (*r*) and the oral morphology (*s*). The variation of these factors with age is considered.

Zusammenfassung—Es wurde beobachtet, daß das Entstehen kariöser Prozesse mit beträchtlicher Regelmäßigkeit in großen Gruppen auftritt. Aber das unregelmäßige Erscheinen von Kavitäten bei Patienten hat zu dem allgemeinen Eindruck geführt, daß Karies zufällig auftritt. Es wurde der Versuch gemacht, die Gruppenregelmäßigkeiten auf den Einzelnen anzuwenden, dabei wurde nur angenommen, daß Karies ein kontinuierlicher Prozeß ist. Unter dieser Annahme ist es möglich, ein Maß für die ätiologische Ursache abzuleiten, das sich unter bestimmten begrenzten Bedingungen mit dem DMF-Wert proportional zeigte. Dies erklärt anscheinend die Tatsache, daß der DMF-Wert auch zum heutigen Kariesauftreten in Beziehung zu bringen ist, obwohl er in früheren Karieserfahrungen begründet ist. Die ätiologischen Faktoren wurden in 3 Gruppen eingeteilt: Speichel (*a*), Zahn (*r*) und Morphologie des Mundes (*s*). Die Veränderungen dieser Faktoren mit dem Alter wurden berücksichtigt.

INTRODUCTION

REPORTS of the incidence of caries are now almost universally presented in terms of the DMF index; such a procedure has been encouraged by several interested organizations, including ORCA. The advantages of the DMF index are simplicity, a distribution which utilizes fairly evenly the entire scale, and good differentiation of experimental alterations.

On the other hand there has resulted a lack of interest in the detailed aspects of caries apparently deriving from the almost exclusive use of the DMF. In any caries survey there results a vast mass of figures which should be capable of yielding valuable information; to condense all this to a series of DMF figures is surely wasteful. Some intermediate stage of calculation which will permit both extraction and understanding of the information is clearly desirable.

The problem may be illustrated in a more specific form by considering some figures provided by a survey carried out several years ago (WALSH and SMART, 1948), which could not have been expressed in terms of the DMF (Fig. 1). This survey was

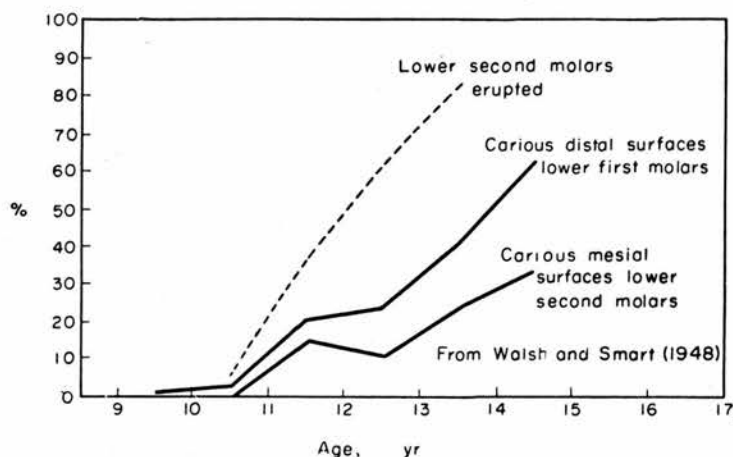


FIG. 1. Percentage of caries on opposing interstitial surfaces of lower first and second molars between the ages of 9 and 14 years. Dotted line indicates percentage of teeth erupted.

chosen because the children show a high level of conservative treatment; qualitatively similar results have been presented by BACKER DIRKS in an accompanying paper. Fig. 1 shows that the distal surface of the first molar develops little caries until the second molar erupts, when it decays rapidly. At the same time the mesial surface of the second molar also decays, but not quite so fast. From these facts one may draw certain conclusions.

The interdental space between the first and second molars is, to a first approximation symmetrical; it follows therefore that the environment and shape of the apposed surfaces are similar. Any difference in the amount of caries in these surfaces can then only be attributed to a difference in the structure of the enamel of the two teeth. This leads to the conclusion that the enamel of the second molar is more resistant to caries than the enamel of the first molar, in spite of the longer exposure of the latter to the oral fluids. One might speculate that this difference is due to the formation of the first molar at a time of greater stress.

This argument is purely qualitative; nevertheless, precise figures are available and it is reasonable to expect that one should be able to devise a quantitative argument leading to a quantitative result. In order to achieve this aim it is first necessary to describe the progress of caries in mathematical terms. A number of equations of this kind are available in the literature, but in each case the interpretation of the parameters in functional terms presents difficulties. The DMF index, on the other

hand, although purely descriptive and unsatisfactory as a mathematical tool seems to bear a remarkably close relationship to a true caries progress function, and the present work was predicated on the supposition that there is in fact a variable which can be used to describe caries progress.

THEORETICAL

There is now little doubt that the appearance of a detectable cavity is merely a stage in the development of a carious lesion. In general the development appears to proceed steadily, although there may be variations in the speed. It may therefore be concluded that the process of formation of a cavity is continuous in the mathematical sense.

It is postulated that the rate of increase in the size of a cavity (G) is equal to the variable (ξ) which it is desired to investigate:

$$\frac{dG}{dt} = \xi. \quad (1)$$

Integration between suitable limits gives:

$$\xi' + \xi_0' = \frac{G}{t - t_e} \quad (2)$$

where t is the age at examination and t_e the age at eruption of the tooth and the dash indicates a mean value over the time interval in question to distinguish the function from the mean value over the population represented by a bar over the symbol. ξ' is the value of the variable attributed to the individual and is equal to zero for the individual having the same amount of caries as the population mean. The instantaneous value of ξ at the time of examination may be obtained from the equation

$$\xi = \xi' + (t - t_e) \frac{d\xi'}{dt}. \quad (3)$$

ξ_0' is the parameter describing the population, which may change with time or with the age of the population, but all values for the same population are comparable. It represents the resultant intensity of the various aetiological factors combined to produce caries. For convenience these factors may be divided into three groups, associated with the activity of the environment (a), the morphological shape of the environment (s) and the resistance of the tooth (r). Thus

$$\xi_0' = f(\bar{a}', \bar{r}', \bar{s}')$$

and

$$\xi' + \xi_0' = f(a', r', s').$$

The exact form of these equations requires rather extensive data for its determination, but it may be expected that a first order relation will be satisfactory initially.

Although experiments can be devised to use G as a variable, in the normal caries survey it is a parameter of the method of examination and may be defined as the number of years required to produce a cavity of just detectable size in a mouth with unit value of the function $(\xi' + \xi'_0)$. It is unlikely that G would show a linear relation with the loss of calcium from the enamel.

RESULTS

To calculate values of ξ' it may be observed that for each value of ξ' there is a corresponding value of t . It follows therefore that the distribution of ξ' will be the same as the distribution of caries in time, and the latter shows an approximately normal distribution. The scale of ξ' can be defined as that which gives a normal distribution of ξ' and thus values of ξ' can be obtained from a table of probits.

Equation (2) predicts that ξ' is inversely proportional to t , so that if ξ' is plotted on a graph against the reciprocal of the exposure time, a straight line should be obtained, provided that the parameters remain constant.

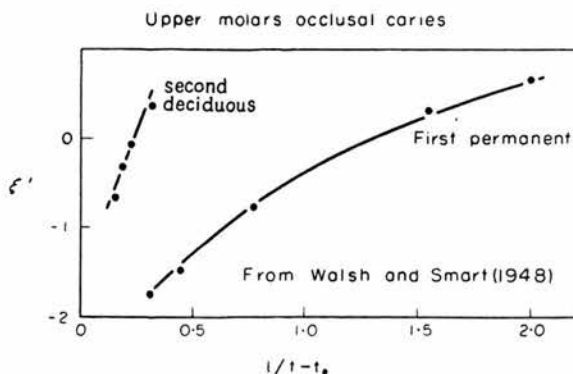


FIG. 2. Relation of ξ' to reciprocal exposure time for occlusal surfaces of first permanent and second deciduous molars between ages of 5 and 8 years.

An example is shown in Fig. 2 for the occlusal surfaces of two teeth at the same ages, but different exposure times. At longer exposure times an approximately linear relation is obtained. This does not prove that ξ'_0 is constant since the value of G probably does not correspond with the slope of the line; the change in ξ'_0 is constant however.

At shorter exposure times a curvature appears which has been found consistently in all figures examined. This curve implies that ξ'_0 shows an initial fall followed by a rise at the same time as the figure for teeth with longer exposure in the same children shows a steady rise. This must be interpreted as a steady increase in the value of \bar{a}' over the period considered (5–8 years), together with a sharp increase in \bar{r}' shortly after eruption tending to a more constant value after a short time.

The relation between ξ' and DMF has been investigated in a number of cases, of which Fig. 3 is an example. There is in general a rough linear relation in each age group, but breaks appear, particularly at DMF 4 and 8, and are more marked if particular types of teeth are considered.

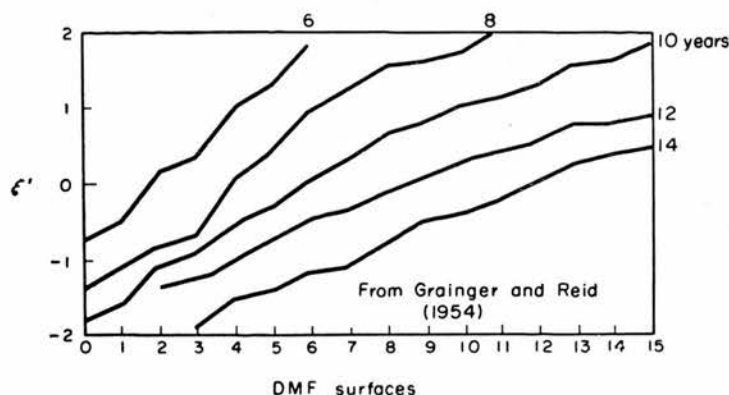


FIG. 3. Relation of ξ' to DMF surfaces between ages of 6 and 14 years.

DISCUSSION

The most pressing need in preventive dentistry at present is a method of evaluating prevention in the individual: to be able to answer the question how much caries would a child have had if he had not been treated with a particular preparation. Such a question cannot be answered by examining the mouth, but will probably require a series of special tests. Nevertheless such tests cannot be relied on in the individual unless some method of assessing individual susceptibility is available, and the relative importance of the various aetiological factors in each individual.

There is however a shortage of suitable figures on which deductions of this kind may be made. Evidence has been presented that there is an increase of enamel resistance after eruption. To give a specific value to this change it is necessary to have two further series of figures. The time of eruption of the carious teeth must be known; this will be earlier than the time of eruption of the whole tooth population, but may be calculated approximately. Secondly it is necessary to have an estimate of the rate of increase in cavity size in order to determine G , although once determined for a particular examiner the value should remain constant.

REFERENCE

WALSH, J. P. and SMART, R. 1948. *N. Z. dent. J.* **44**, 22.

Ultramicro Determination of Phosphate Solubility of Surface Enamel

A method has been described recently for measuring the solubility of the enamel surface in the mouth by recording the time taken for an indicator to change colour in the presence of a small amount of hydrochloric acid¹. It appeared useful to investigate the rate of solution of enamel at a pH closer to that which can be recorded in the mouth and using a more precise estimation. A sensitive method of phosphate determination was developed on the basis of the work of BOLTZ and LUECK² and RHODES³, using reduction of phosphomolybdate in the aqueous phase and extraction of the blue colour produced.

Increased sensitivity was achieved primarily by reduction of the final volume measured, although the solvent causes some enhancement of the absorption. A drift in the spectrophotometer reading (as noted earlier³) was found to be due to water droplets, and could be eliminated by cooling the solution below room temperature and centrifuging.

Using this method the phosphate dissolved in 1 min from the enamel surface of dental patients by 2 μ l of *M* citrate buffer pH 3.5 has been measured. The buffer was applied on filter paper discs, covered on the exposed side with thin polythene sheet to prevent evaporation. A certain proportion of the buffer remained on the tooth when the disc was removed, and it was necessary to add a dye in order to estimate the loss.

Procedure. The tooth was polished with a fine abrasive, washed with water and dried. A 3 mm disc of Whatman no. 4 paper covered with a similar disc of 0.04 mm polythene sheet was saturated with 2 μ l *M* citrate buffer pH 3.5 containing 2% Orange G; the disc was applied to the tooth for 1 min and then placed in 3 ml of water in a stoppered tube. After transportation to the laboratory the dye concentration was determined by measuring the optical density at 480 m μ . 1 ml of a freshly mixed reagent containing 0.8% ammonium molybdate, 4N sulphuric acid and 0.02% hydrazine sulphate was added

and mixed thoroughly. The tube (with a loosely fitting stopper) was placed in a boiling water bath for 15 min and then cooled in ice. The solution was extracted with 1 ml of cooled 4-methyl-2-pentanone and centrifuged briefly at 1000 g. The upper layer was transferred to a 20×3 mm cell and the optical density was measured at 780 m μ . A standard solution was included with each run. The entire chemical procedure apart from the final measurement was carried out in a single tube.

Using the chemical procedure only, a standard deviation of ± 0.13 m μ moles was obtained with a series of known solutions containing 1-9 m μ moles of phosphorus. For repeated determinations of 5 m μ moles the standard deviation was ± 0.36 .

The blank value rose slowly with the age of the reagent, but initially, for discs extracted without contact with a tooth was equivalent to 0.3 m μ moles of phosphorus. The mean amount of phosphorus removed, for all teeth examined in the mouth by this method, and its standard deviation, were 2.3 ± 0.55 m μ moles.

In a series of five central incisors examined in the mouth, the procedure was repeated on the same area, omitting the polishing. The mean value for the first measurement was 2.4 ± 0.6 m μ moles and for the second measurement 1.9 ± 0.2 m μ moles, the variance ratio being statistically significant at the 5% level.

1 m μ mole of phosphorus corresponds approximately to the removal of a 20 Å layer of enamel. The results suggest that the outer 50 Å of enamel is more heterogeneous than the underlying enamel. This may be due in part to the difficulty in controlling the removal of superficial organic matter by polishing.

Résumé. On a développé un ultramicrodosage du phosphore et a appliqué ceci à l'extrait du papier à filtrer qu'on a saturé du tampon critique à pH 3,5 et tenu sur la surface d'une dent dans la bouche.

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¹ T. M. MARTHALER and R. ZILIO, *Helv. odont. Acta* **4**, 27 (1960).

² D. F. BOLTZ and C. H. LUECK, in *Colorimetric Determination of Non-metals* (Ed. by D. F. BOLTZ, Interscience Publishers Inc., New York 1958), p. 29.

³ D. N. RHODES, *Nature (Lond.)* **176**, 215 (1955).